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THE EFFECT OF HEMOGLOBIN ON VOLUME OF THE KIDNEY

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Reid (1929b) found that the intravenous injection of small amounts (5 to 7 cc.) of distilled water caused marked although transient decrease in volume of the kidney. The amount injected did not produce an appreciable alteration in the general blood pressure and but slight variation in the volume of the spleen. Denervation of the kidney did not in any way affect the response to the distilled water. It was therefore evident that the intravenous injection of distilled water produced rather specific peripheral vasoconstriction of the renal vessels. In attempting to determine the mode of action of the distilled water, Reid found that laked erythrocytes in a solution of physiologic sodium chloride produced the same action as distilled water. It was thus indicated that the distilled water destroyed some of the erythrocytes and liberated a substance which caused peripheral vasoconstriction of the renal vessels. Our study was undertaken to attempt to determine which fraction of the laked erythrocytes is responsible for the decrease in volume of the kidney and the site and mode of action.

The fractions of the erythrocytes were prepared as follows: Fresh citrated erythrocytes from dogs were separated from the blood plasma, washed three times with physiologic solution of sodium chloride and laked by the addition of an equal volume of distilled water to which a small amount of ether had been added. The stroma was separated by centrifugation at 30,000 revolutions a minute. The aqueous solution of hemoglobin obtained was chilled to 10°C. and hemoglobin was precipitated by the addition of a saturated solution of ammonium sulphate. The supernatant fluid was removed by passage through a Buchner funnel. The ammonium sulphate which was mixed with the precipitated hemoglobin was removed by dialysis. The solution of pure hemoglobin was

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concentrated in a drying oven. This test hemoglobin was checked by Sheard with the spectrophotometer against known pure hemoglobin, and was found to give the typical absorption spectra. The hemoglobin thus prepared was quite soluble in physiologic solution of sodium chloride and although it cannot be said to be absolutely pure, it seemed to be a trustworthy preparation. Solutions were made up freshly before each experiment.

The stroma was obtained from the centrifuge, washed free of hemoglobin with distilled water, ground in a mortar with sand and sodium chloride and filtered. When physiologic solution of sodium chloride was added opalescence resulted. This was made up freshly for each experiment. All experiments were checked with laked blood. This was prepared freshly, and an equal amount of 2 per cent solution of sodium chloride was added to prevent hemolysis following intravenous injection.

Three series of animal preparations were used. In one series of experiments under ether anesthesia a plethysmograph was applied to the left kidney of a dog, the carotid blood pressure was recorded and the effect of the intravenous injection of the two fractions of the erythrocytes was determined immediately. In another series of experiments the plethysmograph was applied under ether anesthesia, employing sterile technic as described by Reid (1929a). After the animals had been trained to lie quietly, the effect of the intravenous injection of the fractions of the erythrocytes on the volume of the kidney of the intact animal was investigated. Control studies were made in animals in which a plethysmograph had been applied to the spleen in the manner described by Hargis (1926). Finally, kidney preparations from the frog were made for direct observation under low power of the microscope, according to the method described by Richards.

The results of the plethysmograph experiments were conclusive. The intravenous injection of sodium chloride suspension of the stroma of the erythrocytes either did not change the volume of the kidney or it produced a slight increase. The volume of the spleen was not affected except for a slight increase, and the carotid blood pressure did not change. On the other hand, the intravenous injection of hemoglobin dissolved in physiologic solution of sodium chloride produced in every instance a definite, sharp, transient decrease in volume of the kidney. As little as 100 mgm. of hemoglobin produced a distinct decrease in volume of the kidney. Neither blood pressure nor volume of the spleen was altered by the injected hemoglobin. These results were obtained in both the etherized animals and in the trained intact animals (fig. 1, *a*, *b*, and *c*).

The results of the experiment with kidney preparations from the frog were usually not very satisfactory. This was probably mainly due to lack of experience with this type of preparation. However, occasionally

observations were made on the kidney of the frog which compared with the results obtained in the plethysmographic studies. In these preparations low-power fields were selected which showed several working glomeruli, well-filled vessels and a uniform red color of the fields. Following the injection of about 0.2 gram of hemoglobin in 0.5 cc. of 0.4 per cent sodium chloride solution, there was definite slowing of the flow of blood, narrowing

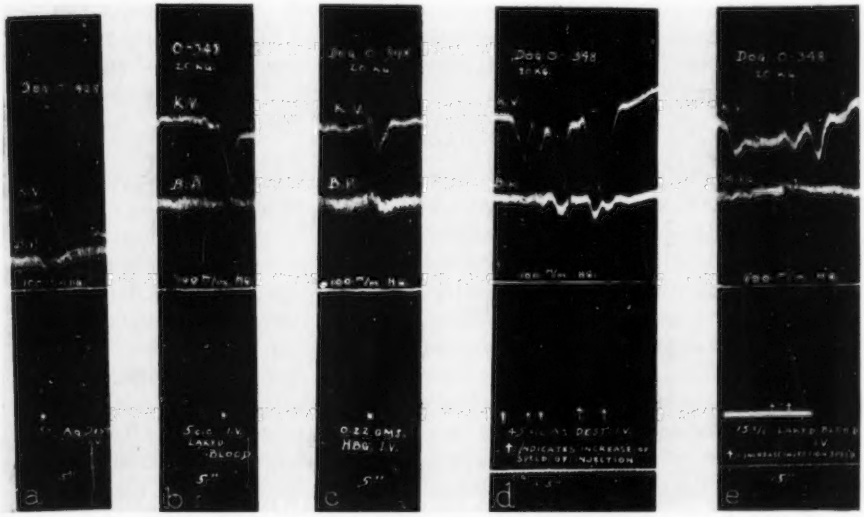

Fig. 1. *a*, kymograph record showing the shrinkage of volume of the kidney without appreciable alteration of blood pressure following intravenous injection of 5 cc. of distilled water; *b*, kymograph record showing the shrinkage of volume of the kidney following the intravenous injection of 5 cc. of laked blood in physiologic solution of sodium chloride; *c*, kymograph record showing the constriction of the volume of the kidney following the intravenous injection of 0.22 gram of hemoglobin, with inappreciable change of blood pressure; *d*, kymograph record showing the multiple shrinkage of volume of the kidney with slight variation of blood pressure produced by increase of speed of injection, during the instillation of 45 cc. of distilled water; *e*, the same type of record as is shown in *d*, with the use of 15 cc. of laked blood as a test substance.

of larger vessels, and disappearance of about half of the working glomeruli. The color of these fields faded to a yellowish-pink. After about two minutes, the caliber of blood vessels was increased, flow of blood became brisk and the color of the fields became red again. There was this difference, however, that the number of working glomeruli, during the observation periods which were limited empirically to two and a half to three minutes, did not return to the number which was present before injection.

Several plethysmographic observations were made in which relatively large volumes of distilled water or of laked blood were injected. This seemed a desirable means of determining the effect on the decrease of volume of the kidney of variation of speed of injection. The doses injected for distilled water were 15 and 45 cc., and for laked blood 20 cc. There was recorded the typical decrease of volume of the kidney, at the onset of injection, following which the volume of the kidney tended to return to the level before injection despite the continued slow injection of the test substances. If at any time during the period of injection the speed of administration was increased, there was a prompt decrease of volume of the kidney (fig. 1, *d* and *e*).

COMMENT

In this study we were chiefly interested in determining which portion of the laked erythrocyte exerted a vasoconstrictor action on the vessels of the kidney. This was accomplished primarily by plethysmographic studies. In this series of observations transient decrease of volume of the kidney was recorded following intravenous injections of distilled water, laked blood, and hemoglobin. The injections of stroma suspension of the erythrocytes produced slight increase of volume of the kidney or were without effect. In the absence of decreases in volume of the spleen, and in view of the inappreciable variations of carotid blood pressure throughout the series of experiments, it may be concluded that the hemoglobin is the constituent of the erythrocytes which exerts a specific vasoconstrictor action on the blood vessels of the kidney. This contraction of the renal vessels was produced whether the hemoglobin itself was introduced into the circulation or whether it was liberated as the result of in vivo laking of erythrocytes.

The minor premise of this work was to obtain data on the mode of action of this vasoconstrictor fraction. On the basis of direct observations of the kidney of the frog, following the intravenous administration of hemoglobin, it was inferred that decrease of volume of the kidney resulted from generalized contraction of renal arterioles.

Further evidence on the mode of action was obtained from plethysmographic records following the injection of relatively large amounts of distilled water and laked blood. In these observations there was always a primary response of the kidney with typical constriction curves and an attempt to recover the volume which was possible before injection, despite slow continued administration of the test substance. Increases of speed of injection were promptly followed by secondary transient decreases of volume of the kidney. This phase of the study seems to indicate that the renal vessels will tolerate a certain amount of free hemoglobin in the blood stream, but that when this amount is increased above a certain

threshold by increased hemolysis or increased amount of injected hemoglobin reaching them, they respond with contraction. One possible source of error must be considered, namely, that the hemoglobin was not absolutely pure and that the active principle was adsorbed or attached by the hemoglobin fraction.

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THE SENSITIZATION BY COCAINE OF GASTRIC AND UTERINE SMOOTH MUSCLE TO THE INHIBITORY ACTION OF ADRENIN

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The literature concerning the effect of cocaine on the action of adrenin on the smooth muscle of the gastro-intestinal tract and on the uterus is contradictory.

In reference to intestinal muscle we find the following widely diverging statements. Lindblom (1926a) reports a sensitization; Thiener and Hockett (1928) deny any sensitization and claim only occasional desensitization; Burn and Tainter (1931) refuse to draw any conclusions, on account of variable results; finally Seidenfeld and Tainter (1931) declare there is neither sensitization nor desensitization.

The same confusing situation exists in regard to the action on the uterus. Lindblom (1926b) again reports a sensitization; Thiener and Hockett (1928) confirm this sensitization even as great as 20 to 400 per cent; Burn and Tainter (1931) find a desensitization.

All the experiments of these authors were made on excised tissues. We thought it would be desirable both to repeat the observations on the isolated muscles and to investigate the behavior under more physiological conditions, that is, with the viscera in situ.

A. Experiments on excised intestine of the rabbit. Magnus' technique was used (Magnus, 1904) with oxygenated mammalian Ringer's solution (150 cc.) for the bath. The temperature was kept constant at 40°C. The pH of the solution (7.4, determined colorimetrically) did not alter on the addition of either cocaine or adrenalin.

Fifteen experiments were made. A first dose of adrenalin was added (from 0.2 to 0.4 cc. of a 1/100,000 solution, giving dilutions of from 1/75,000,000 to 1/37,500,000). This dose gave always a marked fall in the tone of the muscle and a diminution of the amplitude of the contractions.

In some instances the same dose was added after full recovery and in a few cases we confirmed Seidenfeld and Tainter's observation that the second dose had less marked effects than the first one.

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Cocaine was next added in doses varying from 1 to 10 mgm. (dilutions between 1/150,000 and 1/15,000). Cocaine always produced an inhibition in the tone and amplitude of the contractions. The intestine sometimes recovered from this inhibition, and sometimes did not. We discarded these latter cases.

We did not deem it advisable to change the solution for a fresh one, as under these circumstances it is impossible to estimate the amount of cocaine absorbed. Since the intestine was now beating as at first we assumed that the action of the previous dose of adrenalin had passed off.

The same dose of adrenalin was next added anew. The results were varying and contradictory, from a marked desensitization (smaller drop in tone and amplitude of rhythmic contractions and shorter duration of action) to a clear sensitization (sharper fall, smaller contractions and longer period till recovery), with some paradoxical intermediary effects (smaller fall but longer action, or vice versa). In one case cocaine had no effect. These discordant results are in accord with those of the observers cited above.

B. EXPERIMENTS ON THE STOMACH IN SITU. *1. Method.* Cats were used. Food (salmon and milk) was administered approximately one hour before the experiment.

The animals were decerebrated without anesthesia. A cannula was inserted in the trachea for artificial respiration and another in the femoral vein for injections. The splanchnics were cut on both sides by a lateral approach, to eliminate their inhibitory influence on gastric movements. Sometimes both adrenals were ligated by the same route, a procedure which also increased gastric motility.

In some cases the animal had been prepared previously (one or two weeks before) by aseptic section of the splanchnics and removal of one adrenal and denervation of the other, under ether anesthesia.

In two of the experiments both vagi were cut in the neck; this did not interfere with the movements of the stomach.

The stomach was now exposed by a small incision along the midline at the epigastrium, and the shorter branch of a light writing lever was attached by a thread and hook to the peritoneum of the viscus approximately in the region of the pyloric antrum. The lever was adjusted to furnish five-fold amplification at the writing point.

If the stomach is not pulled or twisted during the procedure and if the animal is quiet, save for the respiratory movements, this arrangement registers accurately gastric movements and changes in tone. They appear as primary waves in the graph, on which the respiratory movements are superimposed. It is convenient to use always artificial respiration in order to have a uniform pattern of respiratory waves.

The portion of the stomach wall exposed is so small that shock or loss

of heat is minimal. As an accessory precaution it is convenient to keep the region moist with a few drops of warm Ringer's solution at intervals (3 to 10 minutes).

2. Action of cocaine on gastric movements. It is desirable to use a relatively large amount of cocaine if any influence is to be observed on the action of adrenin. If adequate doses (from 5 to 15 mgm. per kilo) are given *rapidly* the stomach generally enters into a state of complete relaxation and peristalsis will not reappear under the circumstances of the experiment; acetyl choline or pilocarpine are not effective in restoring gastric tone as they may be given only in small doses on account of their circulatory depressor action; stimulation of the vagus, besides checking the heart rate, produces movements (sometimes vomiting) only while the stimulus is being applied. If the cocaine is injected *slowly* enough, however, even the larger doses mentioned above may be given without stopping the gastric peristaltic waves. There is always a slight depression of tone and of amplitude of the waves, but after some time (approximately 15 minutes) the previous condition is recovered.

3. Results. The doses of adrenalin used varied between 0.2 and 1 cc. of a 1/50,000 dilution given intravenously.

Six experiments were made (discounting those discarded because of hyperactivity of the animal after the decerebration or inadequate gastric movements).

The same amount of adrenalin given several times in succession, before cocaine, elicits approximately equal inhibitions, i.e., its effects do not diminish as they sometimes do in the excised muscle.

The same dose of adrenalin given before and after the injection of cocaine produces in the latter case a more marked fall in tone and a longer inhibitory pause. The degree of sensitization by cocaine varies greatly and the number of our experiments is too small to allow a quantitative estimation. In no case was there a desensitization. Figure 1 illustrates a typical sensitization.

C. Experiments on excised uterine strips. The same technique mentioned for the intestinal strips was used. The same amounts of cocaine were added. The doses of adrenalin were higher: 1 to 2 cc. of a 1/100,000 solution (total dilutions in the bath from 1/15,000,000 to 1/7,500,000). Pregnant and non-pregnant cats' and guinea pigs' uteri were tested. In eight experiments six did not show any change in the effect of adrenin after the addition of cocaine, or only a very slight diminution of the response; two showed a slight but clear sensitization. The uteri in these two were from non-pregnant guinea pigs.

D. Experiments on the uterus in situ. *1. Method.* Cats were used, virgin or non-pregnant being preferred.

The animals were decerebrated without anesthesia. A cannula was

inserted in the trachea for artificial respiration and another in the femoral vein for injections.

A median incision was made at the hypogastrium and through it the hypogastric nerves were sectioned. In a few cases the adrenals were ligated by a lateral approach.

The bladder was emptied and brought downward through the wound and held by a clamp. The intestines were tucked upwards and restrained

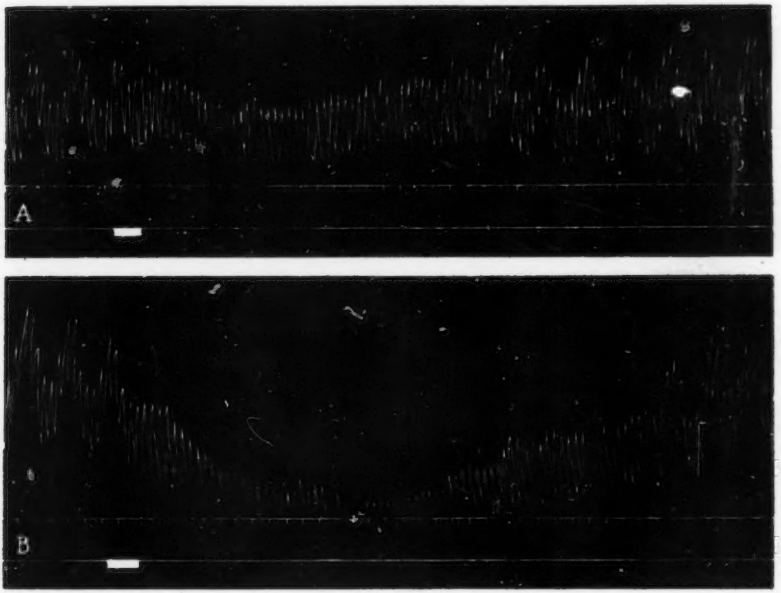

Fig. 1. Effects on the gastric movements (the slow oscillations) of 0.5 cc. adrenalin, 1/50,000, given intravenously at the signal, (A) before and (B) after injecting 10 mgm. cocaine hydrochloride. Weight of the cat, 2.3 kgm. Fed 70 minutes before experiment. Decerebrated. Artificial respiration. Splanchnics cut and adrenals ligated. In this and the other records time is marked in 5 seconds.

with cotton. A hook was now attached to the uterus at the upper part of the body at the angle formed by the junction of the horns, and connected by a thread to the shorter arm of a writing lever which amplified the movements approximately six times. Here, again, the abdominal viscera are so carefully handled and the opening is so small that the animal, which is kept warm by an electric pad, does not present any appreciable shock, as was determined in some instances by registering the blood pressure. The uterus is kept moist by applying at regular intervals a few drops of warm Ringer's solution.

The weight of the lever is adjusted so as to raise slightly the portion of the uterus to which it is attached. Since the uterus contracts usually as a whole, together with the vagina, every contraction raises the writing end of the lever. The respiratory movements are hardly transmitted and in no way hinder the interpretation of the graph.

All uteri, thus prepared, whether pregnant or virgin, contracted rhythmically and regularly from the start.

2. Action of cocaine on uterine movements. As Kuroda (1915) showed, and other authors confirmed, cocaine causes a contraction of excised uterine muscle. The same is true for the uterus in situ, whether it be pregnant or non-pregnant. While it is possible, however, to obtain a permanent contraction of the excised muscle, which, unless the bath be changed for a fresh one without cocaine, will persist until fatigue (i.e., unresponsiveness) appears, this condition is not realized in the intact animal. Here, when the doses of cocaine mentioned before are injected, after some minutes of higher tonus and more frequent and energetic contractions, the previous state usually is recovered. The intensity and length of the stimulating effect depend on the rate of injection; rapid injections increase the effects, slow ones may be hardly noticeable.

3. Results. The doses of adrenalin and of cocaine were the same as those used in the experiments on the gastric movements.

Five experiments were made; four on non-pregnant and one on a pregnant cat.

The same amount of adrenalin given several (2 to 4) times in succession, before any cocaine is administered, elicits approximately equal responses.

In non-pregnant animals, after cocaine has been injected, the same dose of adrenalin produces fairly uniform responses but with greater fall in tonus and a longer inhibitory period. In the pregnant cat the augmentory effects were not clear. The sensitization varied too much and the experiments were too few to allow a quantitative statement. In no case was there a desensitization. Figure 2 illustrates a typical sensitization.

E. Discussion. The contradictory results obtained by experiments performed on the excised strips of intestine and uterus are easily explainable. The authors mentioned adopted, in general, different experimental conditions. The doses of the drugs employed were sometimes widely diverging. The animal species from which the organ was taken was not always the same. This has great importance in a study of the movements of the uterus, as we shall show later.

There are, furthermore, some general criticisms which apply to the method itself. It must not be forgotten that excised tissues are more or less rapidly deteriorating; it cannot, therefore, be surprising that they show abnormal or non-standard reactions. Also, even under apparently uniform experimental conditions, it is impossible to determine or even approx-

imately estimate what doses of the substances employed are actually effective. This will depend on the amount absorbed, and there are too many factors which may influence the absorption,—method used to prepare the organ, handling of the piece, contact with foreign bodies, dimensions of the strip, time elapsed between excision and use, etc. The fact that a second application of adrenalin to the intestinal strip will sometimes produce a feebler response than the first, even though the bath has been changed and the tissue has apparently recovered its former activity, clearly proves that the conditions of the experiment are not uniform.

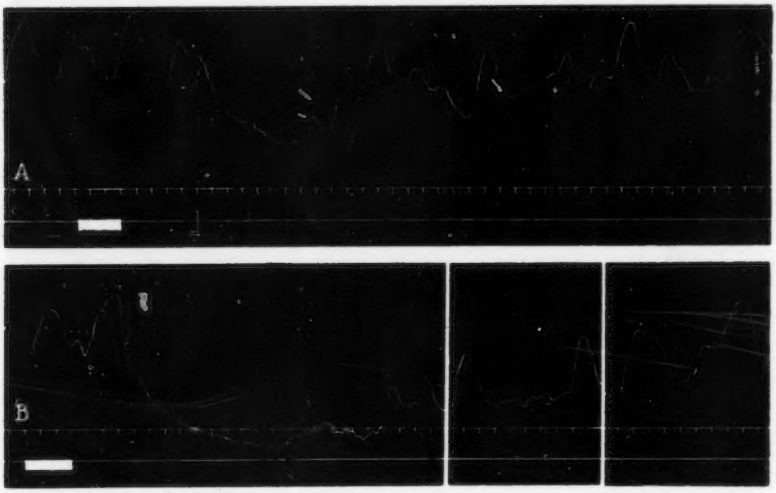

Fig. 2. Effects on the uterine movements of 1 cc. adrenalin, 1/100,000, given intravenously at the signal, (A) before and (B) after injecting 10 mgm cocaine hydrochloride. The record of recovery in B has been shortened by removal of 55 and 75 seconds from the original tracing. Non-pregnant cat, weight, 3.4 kgm. Decerebrated. Artificial respiration. Hypogastrics cut.

We stressed the importance of the animal species from which the uterus is taken. As Dale (1906) and Cushny (1906), independently, first showed, the reactions of diverse uteri to adrenin are different; in the guinea pig, whether pregnant or virgin, there is always an inhibition; in the cat the non-pregnant uterus is inhibited but the pregnant is stimulated; in the rabbit, whether pregnant or virgin, there is always a contraction.

If the cocaine is left in the bath till the second dose of adrenalin is added the muscle does not usually fully recover its first condition, but maintains a more or less contracted state, according to the dose. On the addition of adrenalin we may, therefore, have two forces acting in the same or in op-

posite directions, and our conclusions would not be legitimate if we should disregard the action of cocaine per se.

This view is supported by the results published. Lindblom, and Thiener and Hockett, who reported a sensitization, used rabbits' uteri; in this case, since adrenin produces a contraction, its effect would add to the stimulation by cocaine. Burn and Tainter investigated the behavior of the non-pregnant cat's uterus which is inhibited by adrenin, and found a desensitization; here the actions of adrenin and cocaine are antagonistic.

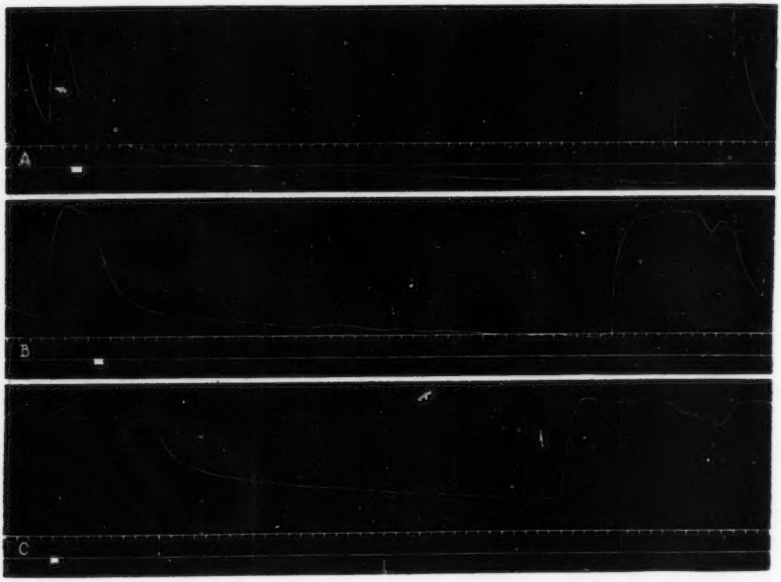

Fig. 3. Effects of 1 cc. adrenalin, 1/50,000, (added to the 150-cc. bath at the signal) on the excised uterus of a pregnant guinea pig, (A) before any cocaine hydrochloride was added, (B) after 2 mgm. cocaine hydrochloride, and (C) after 4 mgm. cocaine hydrochloride.

One of our experiments shows very clearly this play of antagonistic forces; we reproduce it in figure 3. The inhibitory effect of adrenalin was very little smaller, *B*, after 2 mgm. of cocaine were added than before, *A*. In *C* 2 mgm. more of cocaine were given, until a permanent state of contraction was reached; a further dose of adrenalin produced a much shorter and smaller inhibition and the permanent contraction reappeared, only to vanish gradually when fatigue of the muscle set in. We think it would be unjustifiable to speak of a desensitization in such a case.

For all these reasons we think the method is inadequate for the study of this subject and should, therefore, be abandoned.

The experiments on the organs in situ furnish a more favorable and uniform situation, especially when anesthetics are avoided that might interfere with the results, and when the blood pressure is satisfactory. This view is supported by the fact that the same doses of adrenalin, given before the injection of cocaine, will produce equal reactions; likewise, the same doses of adrenalin occasion equal responses, but of a greater magnitude, after cocaine has been administered.

The consistent results of sensitization agree with those previously demonstrated by Fröhlich and Loewi (1910), and other authors, for smooth muscle in blood vessels, iris, bladder. Recent observations here allow the addition of the nictitating membrane.

In these studies we did not find the proportionality of the sensitization to the amount of cocaine injected which has been found in the responses of the blood vessels (Rosenblueth and Schlossberg, 1931). This might depend on the different conditions for absorption of cocaine by the structures involved. The difference is in any case only quantitative, not qualitative.

Sensitization by cocaine appears to differ from the one produced by denervation, since denervation seems to affect only responses from tissues receiving excitatory impulses from the sympathetic and may not sensitize inhibitory influences.

SUMMARY AND CONCLUSIONS

The action of cocaine on the effects produced by adrenin on excised intestinal and uterine muscles was studied. The results were contradictory. The method is criticized and deemed inadequate.

The same action was studied on the cat's stomach and uterus in situ.

Cocaine augments the inhibitory effects of adrenin on the movements of the stomach (see fig. 1) and of the non-pregnant uterus of the cat (see fig. 2) thus sensitizing these smooth muscles as it does all others studied up to the present.

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CONTRIBUTORY FACTORS IN PARATHYROID TETANY IN DOGS

High Temperature, Panting and Overventilation

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The tetany which develops in dogs after removal of the parathyroid glands is generally reported to be more severe in type than in most other animals. If the basic disturbance is the same it follows that the dog is either more dependent upon the presence or proper functioning of the parathyroid tissue than most other animals or that the violent character of the seizures is secondary to some subsidiary factor especially prominent in this animal. That the latter is the case is indicated by the experiments herewith reported. The mechanism involved is related to a rise of temperature, consequent panting and the subsequent development of alkalosis which augments the tetany, which in turn still further raises the temperature with the establishment of a vicious cycle which progressively augments the symptoms and may result in continued convulsions and death unless conservative treatment be resorted to. These conservative measures will be described.

Experiments. *Methods.* A bilateral thyro-parathyroidectomy under ether anesthesia was performed upon forty dogs, weighing from 11 to 30 pounds. These animals were kept without food pending the onset of the characteristic symptoms of tetany or until other considerations made it advisable to place them on special diets.

In the course of the experiments the blood was analyzed for hydrogen ion concentration, carbon dioxide content and tension, serum calcium content; and in some instances for sugar and phosphate content.

The hydrogen ion concentrations were determined by Cullen's (1922) colorimetric method at 38°C. Although the correction which must be employed in this method to obtain the actual pH value is very constant in man, it varies greatly in dogs. As we have been interested in variations rather than actual values we have assumed that this correction remained constant for a given dog and have omitted it. Our values represent

[1] Submitted by W. Ray Bryan to the Graduate School of Vanderbilt University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

therefore, the colorimetric determination at 38°C. rather than the corrected pH values. As this correction is usually less than 0.08 pH when the determinations are made at 38° (Austin, Stadie and Robinson, 1925) our values are within 0.08 pH of the actual pH values.

The total carbon dioxide content of serum was determined by the manometric method of Van Slyke and Neill, (1924). *Serum carbon dioxide tensions* were calculated by means of the Henderson-Hasselbalch equation using the solubility coefficient of carbon dioxide of Van Slyke, Sendroy, Hastings and Neill (1928) and a pK′ of 6.10.

Calcium determinations of serum were made by the Clark and Collip (1925) modification of the Kramer-Tisdall method.

In experiments in which kymographic records of tetany were desired the animals were suspended by a sling in a frame which rested on four rubber bulbs connected to a tambour by means of a rubber tube. *Respiration* was recorded by means of pneumograph.

The blood, unless otherwise designated, was obtained from the superficial veins of the legs. In order to prevent loss of CO_2 the blood samples were taken under oil and then centrifuged under solid paraffin and the serum withdrawn under oil.

Results of type experiments are presented in charts. The temperature curve, expressed in degrees centigrade, represents the actual rectal temperature variations taken with a certified thermometer. The remaining curves represent changes (Δ pH) in pH of serum expressed in hundredths (0.01) pH; changes in carbon dioxide tension (Δ pCO_2) of serum expressed in millimeters of mercury; and changes (Δ $[CO_2]$) in total carbon dioxide concentration of the serum expressed in volumes per cent. As the variations in the acid base condition of the blood are the prime object of these experiments, and since for reasons stated above, the pH values obtained represent the colorimetric determinations at 38°C. rather than the actual pH values, it seems more desirable to plot these curves in terms of variations. The onset, relative changes in severity and cessation of muscular contractions, and panting respectively are indicated by lines of varying width at the bottom of the charts. The zero level of the pH, pCO_2 and $[CO_2]$ curves does not necessarily represent the normal blood level, but the level at which the first sample was obtained. In all charts, however, when the first sample coincides with the first appearance of tetany, the zero level is the same as the normal level. In instances in which the first sample was not obtained until tetany had continued for some time, the zero level on the chart may be slightly below or above the normal level, according to the relative effects of muscular activity and increased respiration.

Results. In earlier experiments we attempted to follow the blood picture at short intervals but were confronted with the difficulty that the with-

drawal of large quantities of blood greatly decreased the severity of tetany. Swingle and Wenner (1926), have shown that hemorrhage is accompanied by an increase in the serum calcium sufficient to account for the alleviation

TABLE 1

Showing little or no variation in pH at the onset of fibrillary twitching from the normal value; and the slightly lower alkali reserve noted at this time

DOG NUMBER	NUMBER OF SAMPLE	DATE	TIME	pH	CO_2	ALKALI RESERVE	REMARKS
					vols. per cent	*vols. per cent*	
III	1	12 /3/29	10:45 a.m.	7.53	56.80	54.81	Normal
	2	12/ 4/29	12:15 a.m	hemolyzed	42.27		25½ hours after operation
	3	12/ 5/29	9:40 a.m.	7.51	48.10	45.22	Onset of fibrillary contractions
VI	1	12/16/29	3:00 p.m.	7.47	53.50	51.31	Normal
	2	12/16/29	3:25 p.m.	7.47	53.40	51.22	Normal
	3	12/17/29	9:00 p.m.	7.48	51.19	49.69	Onset of fibrillary contractions
VII	1	1/ 3/30	10:00 a.m.	7.46	51.07	48.94	Normal
	2	1/ 4/30	10:15 a.m.	7.44	44.97	41.93	Onset of fibrillary contractions
IX	1	1/17/30	4:30 p.m.	7.53	62.20	59.97	Normal
	2	1/18/30	8:45 a.m.	7.50	58.41	54.56	Before operation
	3	1/18/30	4:30 p.m.	7.56	64.18	63.69	7 hours after operation
	4	1/19/30	11:50 a.m.	7.50	53.86	52.25	Onset of fibrillary contractions
XI	1	4/18/30	10:15 a.m.	7.42	51.55	49.20	Normal
	2	4/19/30	9:30 a.m.	7.42	46.10	43.99	Onset of fibrillary contractions
G-I	1	3/17/31	2:30 p.m.	7.48	44.40	42.62	Normal
	2	3/18/31	1:00 p.m.	7.46	46.22	42.76	Onset of fibrillary contractions
G-III	1	3/19/31	10:30 a.m.	7.48			Normal
	2	3/20/31	3:00 a.m.	7.45	47.01	44.40	Onset of fibrillary contractions

of tetany when large quantities of blood are withdrawn. Johnson and Wilson (1929), also found that in unanaesthetized dogs hemorrhage results in an immediate shift toward alkalosis which was followed by a prolonged

decrease in pH. This fact would also tend to reduce the severity of tetany. In view of these findings, and since the acid-base condition of the blood has been shown by many investigators (Hastings and Murray, 1921; Underhill and Nellans, 1921; Henderson, 1920; and others) to be very constant before the onset of tetany, we have followed the blood only during acute attacks.

Table 1 shows seven instances in which we were able to obtain samples of blood just at the onset of tetany. The figures afford substantiating evidence that there is no appreciable change in the pH of the blood following parathyroidectomy until tetany actually appears.

The following experiments show definitely that certain of the phenomena which appear during an acute attack of tetany are so linked as to induce a vicious cycle which progressively augments the severity of tetany, and that the symptoms complex may be altered by modifying any link of this cyclic chain. The experiments were planned to show in the following order, the effects of the increased muscular activity of tetany on temperature, the effect of the temperature on panting and overventilation, and finally the effect of this overventilation on the acid-base balance and tetany.

Hyperpyrexia has been cited by many observers as a symptom which follows parathyroidectomy (Schiff, 1884; Boldyreff, 1908; Dragstedt, 1927; and others). We have observed that at the onset of tetany the temperature level for the individual animal may show no deviation from that under normal conditions, but that as the muscular contractions continue to increase in severity the temperature rises slowly. In most instances where violent tetany was observed there was a definite increase in the severity of the tetany very soon after the dog started to pant. There was also a sharp rise in temperature during this period of increased muscular activity (figs. 1, 2, 4). On the other hand, when tetany was quickly relieved by intravenous injections of calcium lactate the temperature dropped very rapidly after muscular activity ceased (figs. 1, 2, 3); panting continued, however, until the temperature had reached the pre-panting level. We have thus established by repeated experiments that the temperature changes parallel closely the severity of muscular contractions. It is very probable that the high temperature, in some instances as high as 44°C., observed during acute parathyroid tetany is due solely to this muscular activity.

As noted above, in most instances there was a definite increase in the severity of tetany very soon after the dog started to pant. Collip (1926) pointed out that "in about 50 per cent of untreated parathyroidectomized dogs, the outstanding prodromal sign of an approaching tetanic seizure is violent hyperpnea. The animals in this group and manifesting this sign, are subject to tetanic seizures of the most violent sort and death results as a rule earlier than in the other group." We have likewise observed that

tetany is more severe in dogs in which hyperpnea is pronounced. In the case of most animals respiration increases coincidently with the development of tetany and the rise of temperature. This increased respiration has no marked effect on the acid base condition of the blood until after the dog has begun to pant quite vigorously ("violent hyperpnea"). In fact there may be a slight increase in the hydrogen ion concentration of the blood due to lactic acid which is produced by the muscular contractions during this time.

Panting is the normal cooling mechanism of the dog and may become marked with extremely high temperatures. The panting which occurs during tetany is of a degree sufficient to cause an appreciable increase (0.1–0.2) in the pH and a decrease (5–12 mm. Hg) in the carbon dioxide tension

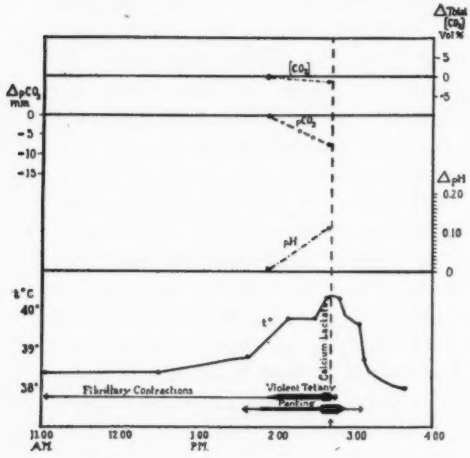

Fig. 1

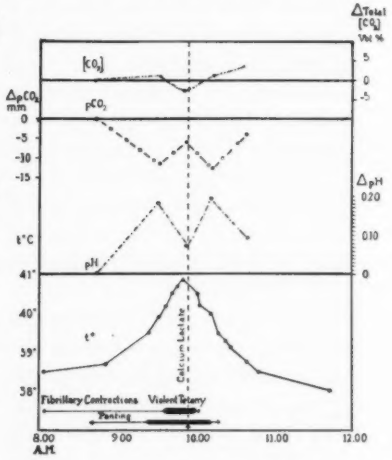

Fig. 2

of the blood. The changes may be obscured in some animals if samples of blood are taken at random, for reasons which will be discussed below. But, if samples of blood taken just at the onset of panting are compared with samples taken soon after panting has become very pronounced, quite appreciable changes are generally observed (figs. 1, 2, 4, 5, 6). In some instances the pH may remain high even during severe tetany in spite of the tendency to acidosis due to muscular activity (fig. 4); in other instances the increased muscular activity, and therefore increased acid production, may temporarily overcome the effects of panting by causing a rapid decrease in the pH and increase in carbon dioxide tension (fig. 1).

The increased alkalinity of the blood during tetany agrees with the work of Steinhaus and Rice (1929) who have shown that in normal dogs the blood

becomes "distinctly alkaline during exercise." They state further that "these changes are related in time and degree to a gradually mounting body temperature."

If samples of blood obtained during severe tetany are compared with samples taken after all muscular activity has been stopped by intravenous injections of calcium lactate, but while the dog still continues to pant vigorously, one again finds quite appreciable changes in the acid-base condition of the blood, i.e., increase in pH and decrease in carbon dioxide tension (figs. 1, 2). Control experiments show that the calcium lactate does not alter the acid base condition of the blood when injected into normal dogs in quantities used in the above experiments (10–20 cc. of 3.8 per cent

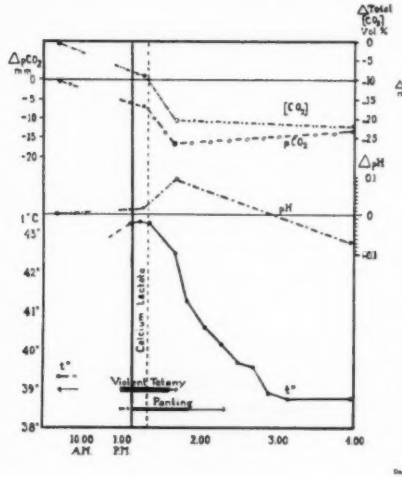

Fig. 3

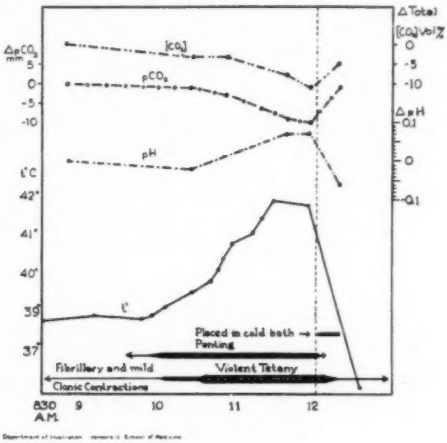

Fig. 4

—isotonic) and since the increased alkalinity disappears after panting ceases (figs. 1, 2) we may conclude that this increased alkalinity is due solely to the panting. Although these changes in the blood picture are of the same magnitude as in the previous instance, tetany does not reappear. With an adequate amount of calcium in the blood the variations in the acid-base condition of the blood due to panting were not of a degree sufficient to cause tetany in any of our experiments.

MacCallum and Voegtlin (1909) first found that parathyroid tetany is augmented by the injection of alkali, i.e., increasing the alkalinity of the blood. Experimental tetany resulting from forced ventilation of the lungs and consequent development of alkalosis has been described by Collip and Backus (1920), and also by Grant and Goldman (1920). As the blood

calcium may be perfectly normal in the tetany of alkalosis the former investigators assume that "the symptoms result from a decrease in calcium ions which is an inevitable result of an increased pH of the blood."

When dogs with low blood calcium and in a condition of hyperexcitability following parathyroidectomy are made to pant by the external application of heat, an increase in the alkalinity of the blood results, followed by the appearance of tetany, which disappears upon recooling.

Figure 5 shows a typical experiment in which a dog in mild tetany was quickly thrown into severe convulsions by heating in a warming chamber. Vigorous panting had continued for about fifteen minutes before the convulsions appeared. A sample of blood taken at this time showed a marked increase (0.12 pH) in pH and decrease (10.92 mm. Hg) in carbon dioxide

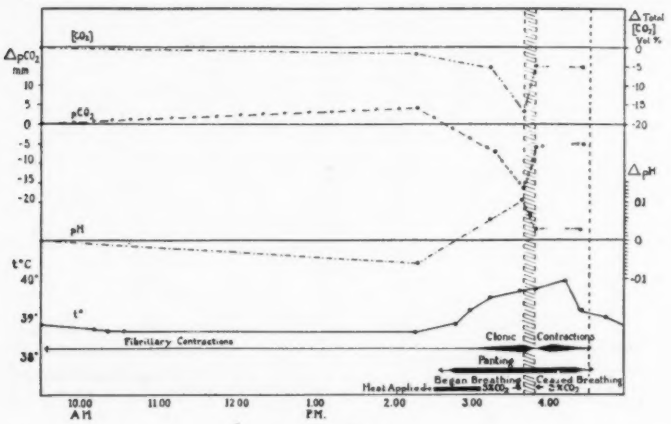

Fig. 5

tension. A few minutes later when the convulsions became quite severe the blood showed an increase of 0.18 pH and a decrease of 20.45 mm. Hg carbon dioxide tension. In this experiment the body temperature having been increased by external applications of heat, the panting and severe convulsions continued, even after the heat was removed, and the body temperature continued to rise. This is an excellent example of the fact that the vicious cycle, once being completed, sustains the convulsive state.

Although some of our animals showed a slightly lowered serum calcium due to parathyroidectomy, the onset of tetany was delayed or did not supervene. We were able to precipitate tetany in many of these animals by subjecting them to heat; the changes in the acid base condition of the blood in the direction and of the magnitude mentioned above were followed by mild clonic contractions. Figure 6 shows a typical experiment of this

type in which mild tetany was precipitated by immersing the dog in a warm bath (43°C.). In this instance the blood calcium determination showed 7.96 mgm. per 100 cc. Vigorous panting had continued for eleven minutes before tetany appeared and a sample of blood taken at the time convulsions were most pronounced showed an increase of 0.24 pH and a decrease of 23 mm. Hg in carbon dioxide tension. The convulsions were not severe enough to maintain the high temperature after the dog was removed from the water bath, and therefore panting and tetany quickly subsided. Attempts to precipitate tetany in this same animal a few weeks later failed even though the alkalosis was more pronounced. The blood calcium at this time was found to be 11.52 mgm. per 100 cc., a reëstablishment of a

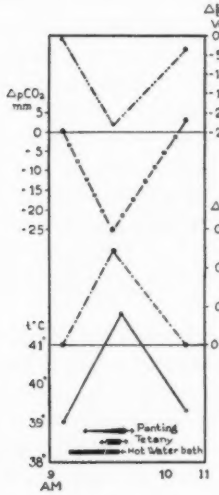

Fig. 6

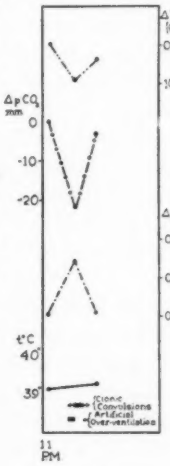

Fig. 7

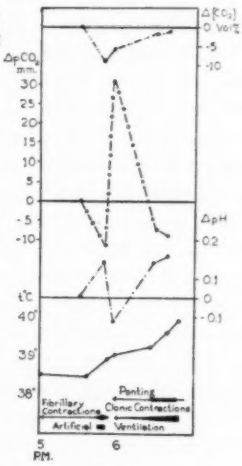

Fig. 8

normal calcium level which may have been due to hypertrophy of accessory parathyroid tissue.

By similar heating we produced corresponding variations of like magnitude in the blood of normal dogs without causing tetany. Rice and Steinhaus (1931) have observed similar changes in the blood of heated normal dogs and in a personal communication Steinhaus states that "overventilation alone does not lead to tetany." In a few normal animals which were overventilated for long periods of time (30 minutes or more) we have observed a slight rigidity of the legs, but have never observed twitchings or clonic jerkings of the muscles in these animals. The pH in one instance was 7.69 when rigidity was observed as compared with a normal pH value of 7.46. The calcium was 11.26 mgm. per 100 cc. serum. These findings

emphasize the fact that the onset of tetany, as well as the augmentation of the symptoms in parathyroidectomized dogs already in tetany, which result when the animals are heated are due to the *summation* of the effects of alkalosis on the condition of hyperexcitability which has been established by the reduction in ionic calcium.

Boldyreff (1908) describes the onset of tetany brought on by heating parathyroidectomized animals, an effect which he attributes directly to the rise of temperature. He made no study of the blood of his animals and hence overlooked the essential factor of an increased pH.

That the tetany is precipitated or augmented by the increased alkalinity of the blood, due to panting, and is not a direct effect of the rise of temperature is shown by the fact that the severe convulsions may be stopped by administering air containing three per cent carbon dioxide and thus decreasing the alkalinity of the blood (fig. 5). By this means the convulsions may be stopped while the temperature remains high. In figure 5 a sample of blood taken at the time severe convulsions ceased showed the blood picture to be practically the same as it was when the convulsions began. The fibrillary twitches, which existed before panting began, were not affected during this short period of CO_2 administration. Swingle, Wenner and Stanley (1927) have shown that parathyroid tetany may be entirely relieved by administration of CO_2. They state that "even the most violent convulsions are rather promptly relieved by slight changes in the reaction of the blood toward the acid side."

Artificial ventilation, without the factor of heat, increases the alkalinity of the blood and precipitates or increases the severity of tetany when the variations are quantitatively comparable to those produced by heat. For this experiment parathyroidectomized animals in the early stages of tetany were barbitalized (200–250 mgm. per kilo body weight) and a tracheal cannula inserted. Vigorous artificial ventilation for two minutes sufficed to precipitate marked tetany as illustrated in figure 7. In this experiment there was an increase of 0.14 pH and a decrease of 21 mm. Hg in the carbon dioxide tension. Tetany ceased within three minutes after overventilation was stopped, accompanied by a return of the blood picture to the previous condition. In figure 8 a dog with a low blood Ca of 6.05 (milligram per 100 cc.), and showing mild fibrillary twitches, was overventilated in like manner with the corresponding precipitation of violent convulsions within two minutes. Similar variations in the acid-base condition of the blood as in the previous experiment were observed; tetany was maintained for five minutes by continuing the overventilation, during which time the body temperature increased from 38.75° to 39.2°C. due to the excessive muscular activity. After overventilation was stopped, there was a short period of apnea, during which there was a sharp decrease in pH and an increase in carbon dioxide tension, and tetany gradually ceased. Owing

to the increased body temperature the dog began to pant, tetany reappeared after three minutes, increased in severity and was accompanied by a rapidly mounting body temperature. A sample of blood taken when violent convulsions reappeared showed a blood picture of alkalosis practically the same as was previously produced artificially. The temperature at this time was 39.9°. Here again we see that the vicious cycle once being completed, tetany continued until it was relieved one hour later by intravenous injection of lactic acid (25 cc., 0.1 N).

When tetany has been increased by panting we have been able uniformly to decrease the severity and restore the animals to their previous condition by cooling them in a water bath (10–20°C.), or by placing them in a refrigerator. The body temperature drops very rapidly under these conditions and panting always decreases markedly or entirely ceases before any change in the severity of the tetany is observed. After panting ceases the tetany decreases and finally reaches the degree of severity that existed before panting began, i.e., only fibriliary twitches or in some instances mild or occasional clonic jerks were present. Figure 4 shows an experiment of this type, in which violent clonic convulsions were quickly relieved by immersing the dog in cool water (11°C.). The temperature dropped rapidly and the alkalosis due to panting disappeared readily after panting ceased, accompanied by a decrease in the severity of tetany. However, fibrillary tremors continued until they were relieved 40 minutes later by intravenous injection of calcium lactate.

Since the blood changes alone suffice to account for the altered symptom complex it seems certain that the direct effects of high body temperatures are of minor significance although they are not entirely ruled out by our experiments. There may also be secondary effects of the increased body temperature which likewise are of minor significance but which must be thought of, such as the effect on the rate of chemical reactions in the tissues, and the effect upon hydrogen ion concentration as pointed out by Austin and Cullen (1926).

Discussion. Panting clearly causes an increase in the severity and in the rapidity of the clonic convulsions and the tonic spasms; the effect on the fibrillary twitches is more difficult to determine. In many animals which show only fibrillary twitches before the onset of panting, violent clonic contractions have appeared soon after panting began (figs. 1, 2, 4). Other animals may show mild or occasional clonic jerks before panting begins (fig. 4). The effect of panting in the latter case is to augment the already existing clonic contractions and in some instances to cause a slight rigidity.

Paton, Findlay and Watson (1916) state that the efferent neurones are the structures primarily implicated in the development of the tremors and jerkings. Carlson and Jacobson (1911) state that their results would

indicate that parathyroidectomy leads "to an increased excitability of the entire nervous system, but that the tremors and tetany symptoms are due to impulses from the brain centers." Even if the efferent neurones are the structures primarily involved there is no doubt that the higher centers of the brain also enter into the picture.

Aside from the factor of overventilation, panting may in another way increase the severity of parathyroid tetany. Blair, King and Garrey (1929) have shown that "the impulses emanating from the respiratory center during inspiration 'irradiate' down the cord. This results in an increase in excitability of the neuro-muscular mechanism manifested by inspiratory augmentation of continuous activities, and of reflexes elicited at the proper time." They further report a "definite inspiratory augmentation of tetany in parathyroidectomies," cf. also King, Blair and Garrey (1931).

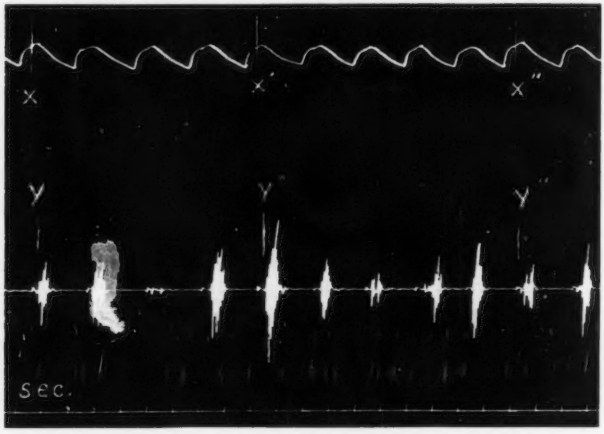

Fig. 9

We have observed four dogs in the course of these experiments in which a definite inspiratory augmentation of tetany occurred. Figure 9 is a graphic record showing marked clonic jerkings with each inspiration. Although the fibrillary twitches were continuous no clonic jerkings were evident between the inspiratory phases.

Other higher centers may contribute to the production of tetany and may be affected, in mueh the same way as they are in shivering, by impulses from the respiratory center, (King, Blair and Garrey, 1931). It may be found that the excessive activity of the respiratory center due to the high temperature, and the consequent "irradiation" of impulses down the cord, is directly responsible for the augmentation of the clonic convulsions during

severe panting, but the factor of overventilation definitely sets the stage for the increased effectiveness of these impulses on the efferent neurone. Conclusive evidence bearing upon these considerations was obtained in experiments in which tetany was augmented or precipitated by the external application of heat; the dogs invariably panted vigorously for from ten to twenty minutes before convulsions appeared. If the convulsions were due solely to the "spilling over" of impulses from the respiratory center they would be expected to appear immediately with the onset of vigorous panting, but this was not the case; for it was only after several minutes of vigorous panting and indeed after a marked increase in the alkalinity of the blood had developed, that these impulses became effective in augmenting tetany. In a similar manner tetany was augmented by vigorous artificial ventilation when the respiratory center was obviously inactive. If thus becomes evident that panting may increase the symptoms of acute tetany by two wholly different mechanisms.

As a result of parathyroidectomy symptoms appear usually in the following sequence: fibrillary tremors or mild clonic jerkings occur, the abnormal muscular activity causes an increase in the body temperature, the dog pants vigorously when the temperature reaches a certain level above normal and the consequent overventilation produces an increase in the alkalinity of the blood. There is thus added to the hyperexcitability already produced by lowered ionic blood calcium, a factor due to increased alkalinity which in some way, perhaps by decreasing the ionization of calcium compounds, augments the severity of the convulsions, which in turn produce a further sharp increase in the body temperature. The panting, therefore, is increased progressively to the ultimate limit. Were it not for the fact that overventilation and alkalosis are opposed by the acid formed in the course of the muscular activity, and in some instances due to decreased ventilation of the lungs resulting from the aspiration of saliva, the vicious cycle of the symptom complex might be expected to result in death within a few minutes.

As it is, one often notes marked periodic variations in the severity during an acute attack of tetany. These variations of tetany are correlated with corresponding rhythmic variations in the pH of the blood, each such variation being self-limited by the mechanism described above and automatically leading to the establishment of the opposite state. Wilson, Stearns and Thurlow (1915) reported a "characteristic rise and fall in the alveolar CO_2 pressure during periods of acute tetany—Soon after the acute attack began, panting commenced and the alveolar CO_2 pressure dropped more or less rapidly to a value below normal. Later there was often a condition of depression and the tremors had nearly or completely disappeared." As was pointed out by Collip (1926), tetany is not nearly so pronounced in dogs which do not manifest hyperpnea. We have also

observed that in the case of animals which do not pant readily the tetany is periodic in character, coming on and disappearing spontaneously at short intervals. Parathyroid tetany, on the whole, is much milder in man and in monkeys (Paton and Findlay, 1916), than in dogs and it is interesting to note in this connection that these animals do not depend upon panting as a cooling mechanism; hence it would be only under exceptional conditions that alkalosis would complicate the picture. Horsley (1885) in describing the effects of thyroid (parathyroid) removal states that muscular twitches of the fibrillary type are the predominant symptoms in the monkey; he also states that he has never observed high temperature in this animal, and further that "the skin remained averagely moist during tetany." On the other hand in the dog, which pants in order to regulate the body temperature, we recognize the more severe, even violent clonic convulsions during the attack as due to the alkalosis. If overventilation does not predominate but is of a milder type, the overventilation merely prolongs the acute attack which, due to the accumulation of acid, would be self limited.

Cullen and Earle (1929) have shown that in normal men the pH of the blood may fluctuate as much as 0.01 pH to 0.07 pH in the course of a single day. These variations may be of considerable importance in the appearance and disappearance of tetany.

The relation of high temperature, panting and overventilation to the symptomatology of tetany in parathyroidectomized dogs, which has been described in the preceding pages, would appear to offer a plausible and satisfactory explanation of the severity of the tetany and the high death rate of such animals during the hot weather of the summer months. A like explanation is suggested for the prompt precipitation of convulsions in the conditions of latent tetany when the animals exercise to the point of severe panting, especially in hot weather.

SUMMARY

1. Evidence is presented which corroborates the view that, following parathyroidectomy in dogs, there is no appreciable variation in the pH of the blood until tetany actually develops.

2. The body temperature is shown to rise slowly with the development of fibrillary contractions and rapidly after the onset of violent tetanic attacks. In extreme instances the body temperature may reach 44°C.

3. A definite shift toward alkalinity, i.e., increased pH and decreased carbon dioxide tension, of the blood develops as a result of overventilation due to the panting which accompanies the rise in body temperature during the acute attacks.

4. In parathyroidectomized dogs the existing hyperexcitability due to low blood calcium, is augmented by increased alkalinity due to panting.

These summated conditions may precipitate attacks or markedly intensify attacks in progress.

5. An equivalent or even greater acid-base change accompanying a rise in temperature and panting of otherwise normal dogs does not precipitate tetanic attacks.

6. The acid products of muscular activity during a tetanic attack may temporarily overcome the decreased hydrogen ion concentration due to overventilation and may thus cause a temporary decrease in the severity of the attack; this phase is followed by an exacerbation of the attack when the increased pH of overventilation again predominates.

7. The augmentation of parathyroid tetany during the inspiratory phase of respiration is confirmed.

8. The important components in the symptom complex after parathyroidectomy in dogs develop in the following sequence: fibrillary muscular contractions, a rise of body temperature, which in turn causes panting and results in overventilation and increased pH. Violent tetany ensues and further augments the rise of temperature. There is thus instituted a vicious cycle which progressively increases the severity of the attack unless relief is obtained by suppression of one or more of the contributory factors.

Indebtedness for the helpful coöperation of Prof. G. E. Cullen is herewith acknowledged.

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ON THE MECHANISM OF OVULATION IN THE RABBIT

III. The Fate of Mechanically Ruptured Follicles

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At the present time it is quite generally agreed that the agent responsible for ovulation is humoral and has its origin in the anterior lobe of the pituitary. There is, however, no general agreement as to the nature of the agent responsible for the luteinisation which follows ovulation. Some workers claim to have separated a follicular growth substance and a luteinising substance from anterior lobe, urine of pregnancy, and placenta (Collip, 1930; Wiesner, 1930; Claus, 1931). It is inferred from such work that following the act of ovulation, the process of luteinisation is effected by a special luteinising hormone. Such inference, to be sure, is not entirely without support Yet, there are at hand data which would make one hesitate to accept as a demonstrated fact the existence of a luteinising hormone, separate and distinct from the hormonal agency which is responsible for ovulation (Friedman, 1930). In the normal animal ovulation does not occur without the subsequent luteinisation of the discharged follicles. Up to the present time no one has succeeded in preparing an extract which produces ovulation without the subsequent luteinisation of the *artificially* discharged follicles. So far as is known, therefore, luteinisation is an inevitable sequel, although not necessarily a consequence, of ovulation. It is, of course, possible that ovulation and luteinisation are effected by different agents despite the regularity of the sequence. But until a separation of the phenomena in question can be demonstrated in vivo, using unaltered the humoral agencies of an intact animal, one must seriously consider the possibility that the act of ovulation is the cause of the process of luteinisation.

Unknowingly, Bouin and Ancel (1909) performed some experiments which might throw some light on the question. These workers, interested then in the functions of the corpus luteum, pricked open the ripe follicles in one ovary of each of several rabbits. They reported that such artificial rupture leads to the formation of corpora lutea not only in the pricked follicles, but also in the unruptured follicles of the same ovary and in the follicles of the ovary on the opposite side. O'Donoghue (1913) repeated

and confirmed the results of Bouin and Ancel. Insofar as the hormone responsible for ovulation is presumably not present in the blood of unmated rabbits in effective concentrations one might conclude from the experiments of Ancel and Bouin and O'Donoghue that either the mechanical rupture of the follicles was itself a sufficient stimulus for the process of luteinisation even in the absence of the usual stimulus for ovulation, or, that the operative procedure led to the liberation of the agent normally responsible for ovulation. From the data of these early workers it is impossible to decide between these possibilities.

Recently Walton and Hammond (1928) repeated the experiments of Bouin and Ancel but were unable to confirm the results of the earlier investigators. In no instance did the artificial rupture of follicles in one ovary of the rabbit lead to the development of corpora lutea either in that ovary or in the ovary of the opposite side.

If one carefully examines the reports of the three series of experiments it is not difficult to find some possible reasons for the discrepancy in the results. In the first place Bouin and Ancel do not tell just how carefully their female rabbits were isolated. To be sure they were kept from the males, but no mention is made of whether or not the animals were isolated from other females. Unless this was done, the corpora lutea found by Bouin and Ancel after their operative interference are of little consequence inasmuch as ovulation is known to occur when a rabbit doe in heat is hopped by another (Hammond and Marshall). O'Donoghue's report is a brief one, entirely lacking in details about the methods used. Walton and Hammond on the other hand, do not indicate that they were certain that their experimental animals were in heat at the time of operation. Bouin and Ancel emphasize the fact that in order to obtain satisfactory results with the procedure they describe, the experimental animals must be in heat and exhibit ripe follicles in the ovaries. Moreover, the number of animals used by Walton and Hammond is small, and it is possible that they did not control sufficiently the factor of operative trauma. In all they punctured the follicles in the ovaries of only four rabbits. Histological section of some of these follicles showed no trace of granulosa remaining. As controls on the factor of operative trauma Walton and Hammond used only two rabbits. In these animals follicular puncture was performed a few hours after coitus. Inasmuch as corpora lutea formed in the operated ovary of one of the two control rabbits, the authors concluded that the factor of operative trauma could not explain their negative results.

In view of the great significance of these experiments in the interpretation of our newly gained data on the pituitary-gonadal relationship, it was decided to repeat the experiments under discussion, controlling as much as possible those factors which might account for the discrepancies in the reported results.

EXPERIMENTAL PROCEDURES AND RESULTS. All animals used in these experiments were maintained in individual cages on a diet of clover hay, oats, carrots, and cabbage. Contact with males or with other females was never allowed except in those instances when matings were attempted. At such times the female was placed with a fertile male and observed. If the female did not accept the male within a period of ten minutes she was returned to her cage and kept isolated until another mating was attempted. If the female did accept the male, she was immediately returned to her individual cage where she was kept isolated for the remainder of the period of experimentation.

SERIES I. *The mechanical rupture of follicles.* Of the eight rabbits selected for this series of experiments, five were pregnant and three had had infertile coitus about one month previously. As soon as the five pregnant females dropped their litters the young were immediately removed. Two to four days later (on the second to fourth days post partum) each of these five females was subjected to operation. The three remaining rabbits were subjected to the operation on the 34th day following the infertile coitus.

The operation was done under ether anesthesia following a preliminary injection of atropine sulphate. Through a ventral flank incision one ovary was exposed and every one of the large follicles, and each of the moderately sized follicles was punctured with a no. 23 hypodermic needle (outside diameter 0.4 mm.), so that in the exposed ovary no follicle having a diameter of approximately one half millimeter or more was left unruptured.

At the time of operation it was noted that the operated ovary in each of these eight animals was quite normal and possessed several large, apparently ripe, follicles. The puncture always occasioned the escape of some follicular fluid, and rather rarely, some slight follicular hemorrhage.

Three to six days following the operation the animals were sacrificed and the ovaries preserved for microscopic study. Upon gross examination no corpora lutea were seen either in the operated or control ovary of any of these eight animals. Microscopically the control ovaries showed large, unaltered follicles. In the operated ovary, follicles were seen in which the ovum was distinctly displaced. In a few follicles a slight hemorrhage was noted. In no case, however, was the granulosa dislodged. Frequently, but not always, the site of the puncture could be clearly seen. A thorough examination of the walls of the punctured follicles revealed no trace of luteinisation

SERIES II. *The mechanical rupture of follicles at varyng intervals after coitus.* In order to evaluate the factor of operative trauma in the first series of experiments, the follicles of one ovary were punctured in each of several rabbits at varying intervals after coitus. For the most part, the rabbits used in these experiments had not been pregnant recently.

In ten females the follicles of one ovary were punctured within thirty

minutes after coitus. In eight of the ten cases etherisation was started within five minutes after coitus, and the follicles were punctured within twenty minutes after coitus. In five other rabbits the operation was not performed until two to three hours after coitus. With two exceptions, the animals were sacrificed 48 hours after coitus, and the ovaries examined grossly and microscopically.

In seven of the ten rabbits operated within thirty minutes of coitus, ovulation failed to occur from either the operated or the unoperated ovary. Of the three rabbits of this group which did ovulate from the untreated ovary, two showed definite luteinisation of the punctured follicles, although the ova were retained (fig. 1). The third rabbit in which ovula-

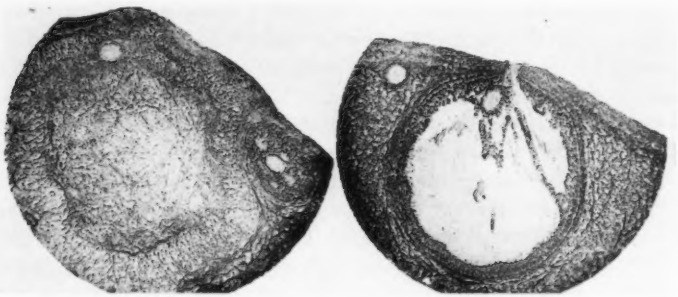

Fig. 1 Fig. 2

Fig. 1. Microphotograph of punctured follicle 48 hours post coitum, showing luteinisation of the follicular wall, and showing the retained ovum near the free surface. Ovulation had occurred from the follicles in the contralateral (untreated) ovary.

Fig. 2. A punctured follicle from an animal in which ovulation from the contralateral (untreated) ovary did not occur. The ovum is definitely displaced; the point of puncture is clearly visible. The granulosa is intact. No luteinsation is seen.

tion occurred from the untreated ovary was sacrificed 24 hours after coitus. This was too early for definite lutein changes to be noted, but other significant changes were detected in the operated ovary of this animal. One of the punctured follicles increased about three times in diameter (to 3.5 mm.). In this greatly enlarged follicle there was some hemorrhage, but the granulosa was not destroyed. The ovum, though definitely displaced, was retained within the follicle. Another follicle in the operated ovary showed those changes which are typical of a recently discharged follicle, and in this case ovulation had presumably occurred. The remaining follicles in this ovary were unchanged except for the obvious defect in the wall caused by the puncture.

The follicles of the unoperated ovary in the seven animals which failed to ovulate could not be distinguished from the ripe follicles of an animal in heat which had not been allowed coitus. The punctured follicles in the operated ovaries of these seven animals showed no detectable changes except those which resulted from the operation. In figure 2 is shown a punctured follicle from the operated ovary of one of these animals to illustrate the amount of damage caused by the operation. This follicle is shown in preference to one from an ovary of an animal in the first series of experiments (in which coitus was not allowed) because this happens to be the only instance in which the plane of the puncture and the plane of the ovum happened to coincide.

In the five animals in which the operation was performed two to three hours after coitus, ovulation from the untreated ovary occurred in every instance. In each of the five animals the punctured follicles became luteinised although the ovum was retained.

Discussion. The negative results of the foregoing experiments are entirely in accord with the findings of Walton and Hammond, and are quite opposed to the results of Bouin and Ancel and of O'Donoghue. It may be recalled that the procedures of Walton and Hammond were possibly subject to criticism on two points; in the first place, because they had not made certain that their experimental animals were in heat, and secondly, because the number of control experiments was not adequate to evaluate properly the factor of operative trauma.

In my opinion such criticisms have been met in the experiments just reported. Insofar as female rabbits regularly come on heat shortly after parturition and are almost invariably fertile at such time if not allowed to suckle their young (Hammond and Marshall), it might justly be inferred that at least five of the eight animals used in the first series of experiments were in heat at the time of operation. Moreover, it does not seem possible to account for the negative results in these experiments on the basis of operative trauma. It is admitted that almost half of the rabbits in the second series of experiments failed to ovulate after the operative procedure which shortly followed coitus. It is barely possible that the combined operative procedures decreased the incidence of ovulation in these animals. Even in untreated animals, however, ovulation does not invariably follow coitus if the females have not recenty been pregnant. Since the rabbits used in the second series of experiments had not recently been pregnant, it is difficult to judge whether or not the operative procedures had any effect on the incidence of ovulation. Regardless of this, however, it is significant that in those eight rabbits of the second series in which the untreated ovary gave evidence of the presence of an adequate stimulus for ovulation, the punctured follicles in the treated ovary of these animals gave equally clear evidence of the presence of the luteinising agent. It

cannot be said, therefore, that the operative trauma in these experiments was so great as to prevent the luteinisation of the punctured follicles even in the presence of an adequate stimulus.

It is interesting to note that ovulation rarely occurred from the punctured follicles despite the presence of an adequate stimulus for ovulation. Instead, they formed partially luteinised structures with retained ova. It is possible that following coitus there are present in the blood of the rabbit two hormones; one, an ovulation provoking substance, and the other, a luteinizing substance. If this were true, one might explain the results obtained in these experiments by assuming that the operative trauma was sufficient to prevent the action of the ovulation provoking substance, but was insufficient to prevent the action of the luteinising principle. It is just as reasonable, however, that the phenomena of ovulation and luteinisation are effected by one hormone, and that the operative procedure prevented the manifestation of but one of the effects of this hormone.

Quite in harmony wih this latter hypothesis are the recent results of Hill and Parkes (1930). These authors report that after ovulation in the rabbit is induced by the single intravenous injection of an extract of urine from a pregnant woman, the discharged follicles develop into normal functioning corpora lutea which endure as long as the corpora lutea which result from sterile coitus. The fact that the corpora resulting from the single injection persisted for about twenty days without further injection, led Hill and Parkes to conclude that "the stimulus to luteinisation of the ruptured follicle is initiated by the actual act of ovulation" . . . . and that the stimulus for ovulation "is not connected with the subsequent release from the pituitary of the stimulus to luteinisation."

In order to accept the conclusions of Hill and Parkes one must assume that the material injected in their experiments was quickly dissipated and that following ovulation the material was no longer available to the ovary. Furthermore, one must also assume that the injection of the material in question did not stimulate some other tissue to set free a luteinising principle. At the present time it seems hardly wise to make either of these two assumptions, although it has recently been shown that extracts of urine of pregnancy are capable or producing luteinisation when injected directly into the ripe follicles of one ovary (Friedman, 1931). The absence of any change in the untreated ovary of the opposite side is clear evidence that the result is due to the direct action of the material on the follicle and not due to some indirect humoral change. Yet even this demonstration does not answer the question at issue. One still must ask whether or not the agents responsible for ovulation and luteinisation are distinct and different.

Only one thing becomes evident . . . mere rupture of a follicle is in itself not sufficient to cause luteinisation, and the phenomenon of luteinisation cannot be regarded as a simple reaction to injury or trauma occasioned by the sudden rupture of the follicular wall.

SUMMARY

1. In eight unmated rabbits the mechanical rupture of the large follicles of one ovary did not lead to the formation of corpora lutea either in the operated ovary or in the contralateral ovary.

2. The trauma of follicular puncture, as practised in these experiments, is not sufficient to prevent the process of luteinisation when an adequate stimulus for the process is present.

3. In the light of these results, it becomes evident that mere follicular rupture is in itself not sufficient to cause luteinisation.

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ON THE RELATION OF BLOOD SUGAR TO BLOOD VOLUME, AND CARBOHYDRATE TO WATER RETENTION

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It has long been known that eating carbohydrate causes water retention in the body and that diabetes is associated with a train of evils, but clinical observations on the relation of carbohydrate to local edemas in non-diabetics have not been followed up by exhaustive biochemical studies (compare Urbach and Sicher). Some biopsies of the skin have been made (Pillsbury, Arndt, Urbach and Fantl, Urbach and Sicher, Nathan and Stern) but they result in scars. It seems probable that storage of sugar and glycogen in the skin and subcutaneous tissue results in water retention in the same tissue. Perhaps everyone has felt the effects of overeating carbohydrate at some Thanksgiving or Christmas dinner but such experiences are soon forgotten.

METHODS. Insensible perspiration was determined by a balance capable of weighing a man to within 10 grams and it was found that insensible perspiration of a man doing work at his desk in a biochemical laboratory at 23° (compare Rogers and Lackey) was of the order of magnitude of 50 grams per hour. The minimum urinary secretion was of the same order of magnitude so that fasting and thirsting might result in a loss of weight of the order of magnitude of 100 grams per hour (in one case 75 grams). Therefore, in studies of the effect of glucose on water retention, at least 100 grams H_2O per hour were drunk. Doctor McQuarrie suggested that isotonic glucose be given but the subjects refused to take the requisite quantity of water every hour and the glucose was made isotonic for the first dose only.

Finger blood sugar was determined in unlaked blood by the Folin and Svedberg modification of the method of Folin and Malmros, using 0.02 cc. of blood, as described by McClendon. Each analysis was carried through without delay at any step and duplicate blood samples were taken.

Blood volume changes were determined by the recent Bausch and Lomb hemoglobin method. It is well known that the hemoglobin content of the blood in the spleen is different from that in the circulation and that changes in volume of the spleen vitiate the method. But the changes in blood

volume observed were much greater than could be accounted for by changes in splenic volume. Furthermore, there is no more accurate method for determining changes in blood volume than by hemoglobin determinations. Rapid changes in blood volume are accounted for largely by changes in water content of the plasma although glucose, protein, salts and other substances may enter and leave the blood stream. Exact correlation between blood sugar and blood volume after the first dose of glucose as recorded by Marx were not observed in all cases. It appeared that some other factors than blood sugar caused small temporary changes in blood volume and only very great changes in blood volume were considered significant.

EXPERIMENTS. As control experiments men were fasted and thirsted on certain days, and blood sugar and volume determined. Small changes in both values were noted and such changes showed no correlation. For instance, in one man there was in 2½ hours a fall of 19 per cent in blood sugar and an increase of 10 per cent in blood volume. The fall in blood sugar may have been due to fasting. During this period there was a loss by insensible perspiration of 40 grams per hour and a urinary secretion of 40 grams per hour, resulting in a loss of water from the body of 200 grams during the period. The increase in blood volume may have been a reversal of a previous *decrease* due to the exercise and cold, incident to a walk of five blocks to the laboratory. Marx obtained rise in blood sugar on drinking water and such rise might antagonize the fall seen in fasting, as shown in the following experiment: A man ate nothing all day but drank a litre of water on arriving at the laboratory after a walk of five blocks. The blood sugar remained constant within the experimental error but the blood volume increased 20 per cent in 2½ hours (compare Brahn and Bielschowsky). There were 1100 cc. urine excreted in 3 hours or a total loss of all the water drunk. For these reasons changes of 20 per cent in blood sugar or in blood volume are not considered significant.

Marx obtained decrease in blood sugar and blood volume on giving insulin. In the present study, however, the blood sugar was lowered with insulin to 35 mgm. per 100 cc. of blood without marked lowering of blood volume. Since twitchings of the muscles occurred, it was not thought safe to give larger doses of insulin to fasting non-diabetics. The lower half of figure 1 (with per cent change shown on ordinate and hours on abscissa) shows average values of blood sugar and volume changes on giving 20 units of insulin to a fasting man. The heavy curved line shows that the blood sugar suffered a fall of more than 50 per cent whereas the light line shows that the blood volume did not fall significantly. Since there was a loss of more than 600 grams water by insensible perspiration and urination during this period of 8 hours, the slight fall in blood volume might be considered as due to dehydration of the body.

The results on ingestion of glucose are more striking as shown in the upper part of figure 1 (heavy line average values for glucose and light line blood volume changes). At the beginning of each experiment, approximately a litre of isotonic glucose solution (50 grams glucose and 1 kgm. water) was drunk and at the end of each hour 50 grams glucose and 200 grams water. The first dose of glucose solution caused the characteristic rise in blood sugar of the ordinary glucose-tolerance-test but no significant change in blood volume. The blood sugar began to fall in a little more

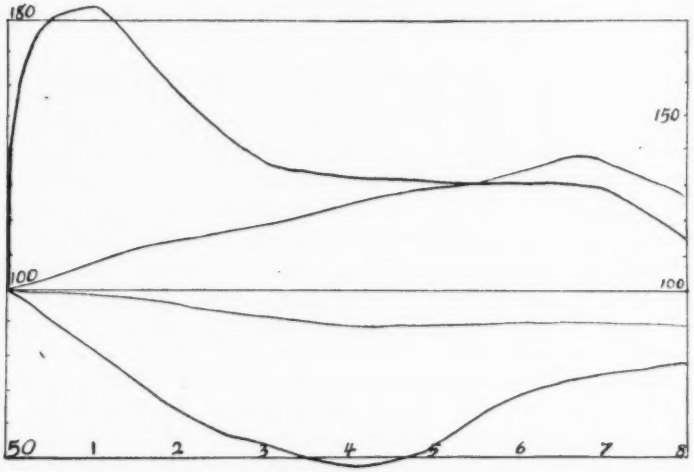

Fig. 1. Changes in blood volume in relation to blood sugar. Per cent change is shown on the ordinate and hours on the abscissa. Blood sugar curves are heavy lines and blood volume curves light lines. The normal, or 100 per cent, is marked by horizontal line, dividing the figure into two parts. The lower part represents average values of fasting and thirsting men given 20 units of insulin at the start. The upper part of the figure represents average values of men given 50 grams of glucose in a litre of water at the start and 50 grams of glucose in 200 cc. of water each hour following.

than an hour despite repeated doses of glucose but the blood volume continued to rise to more than 30 per cent above the normal in all normal men on which it was tried about the end of the seventh hour. If the insensible perspiration and urination are subtracted from the water drunk plus the metabolic water (metabolism calculated by the method of Benedict and Root), the retention of water in the body can all be accounted for by the increase in blood volume. The increase in body weight during such an eight-hour experiment averaged about 1 kgm.

SUMMARY

In experiments on normal men, drinking water while fasting resulted in only small changes in blood sugar and blood volume, and all the water was excreted in a few hours. Taking glucose with the water (400 grams glucose in 2400 grams water in eight hours) resulted in marked increase in blood volume and water retention and increase of about 1 kgm. in body weight.

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THE EFFFCT OF FIBERS OF SPECIFIC TYPES IN THE VAGUS AND SYMPATHETIC NERVES ON THE SINUS AND ATRIUM OF THE TURTLE AND FROG HEART

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The functions served by different fiber types in a mixed nerve have heretofore been determined from the results of artificial stimulation of the nerve trunk as a whole, from its section, or from experiments designed to determine the source of origin of its fibers. These methods have certain obvious limitations. It is felt that recent studies (Bishop and Heinbecker, 1930), in which it has been shown that a correlation can be established between the form of the conducted potential of a nerve and its histological structure, make available another method whereby the function of specific types may be determined with considerable definiteness. Such a potential analysis and fiber correlation has been reported (Heinbecker, 1930) for the cervical sympathetic and vagus trunks of the turtle and cat. Similar findings have been found by Bishop and Heinbecker to obtain in corresponding nerves of other animals including monkey and man.

The object of this paper is to furnish evidence as to the function of fiber types giving rise to certain potential components in the vagus and sympathetic nerves supplying the sinus and atrium of the turtle and bull frog hearts. The similarity of potential form and histological structure of the corresponding nerves of higher animals to those here specifically studied seems to warrant the inference that the present result will be found generally applicable. However, the necessity of caution in making any such generalization is fully appreciated.

MATERIAL AND METHOD. The turtles used were the Pseudemys elegans, P. concinna and P. scripta. The results in all were found similar. In turtle experiments both vagus nerves were dissected free from high in the neck to well down into the body cavity. In most experiments the sinus and atrium with the attached vagus nerves were removed from the body and mounted on a cork board within the incubator in a manner permitting the nerves to be placed on the stimulating and recording electrodes leading to the oscillograph. Each preparation was so arranged that a spring heart lever attached to one atrium recorded the contractions on a kymograph. Simultaneous neurograms and myograms could therefore be made. The

nerves were stimulated by condenser charges. The recording mechanism was the cathode ray oscillograph. By observing the neurogram on the face of the oscillograph it was possible to observe threshold and maximal responses of the potential components and actually correlate potential form with functional activity in the sinus and atrium. Similar experiments were carried out in the body with an intact circulation with identical results.

In bull frog preparations the extremities and all viscera except those in the thorax were cut away. The sympathetic chain was found and the ganglia corresponding to the 3rd and 4th spinal nerves were identified. No attempt was made to separate the ganglia from these nerves. The latter were cut short proximally and distally and the cathode of the stimulating electrodes applied to the branches leading from the ganglia to the heart. The vagus nerve was also freed sufficiently to stimulate. The ventricle was severed from the atrium. The atrial contractions were recorded by the spring heart lever. No attempt was made to perfuse the atrium.

ANALYSIS OF TURTLE VAGUS POTENTIAL. For convenience of reference the potentials derived from the myelinated fibers of the vagus are referred to as B_1, B_2 and B_3, the potential from the unmyelinated fibers as C (Heinbecker, loc. cit.). As it was necessary to determine whether or not the strength required for a maximum response of certain fiber groups in the turtle vagus was greater than that required for a threshold response of the next less irritable group the potential of the turtle vagus nerve was analysed in detail. The results appear in table 1 and figure 1. They indicate that it is frequently impossible to stimulate all of the B_2 fiber group before some of the C fibers are stimulated. However it is possible to see definitely the development of each of the potential elements on the face of the oscillograph and to correlate the observations with the functional state. The overlapping of potential groups by thresholds is stressed because of the importance of the recognition of this fact in any attempt to explain the findings.

RESULTS. Stimulation peripherally with stimuli adequate to elicit only the B_2 and preceding potentials was invariably without effect on the atrium. Evidence that the B_1 potential arises from afferent fibers has been presented elsewhere (Heinbecker, 1930). The B_2 fibers are considered efferent but if any of them innervate the heart, their stimulation does not cause any of the effects here studied. At the first sign of the B_3 potential there occurs a lowering of the amplitude of contraction together with a shortening of systolic time *but no change in rate* (figs. 2 and 3). On increasing the area of this potential by strengthening the stimulus employed the effects are increased. Only when the stimulus strength is adequate to elicit the *first sign* of a "C" potential does there occur a first slowing of the heart rate. If the frequency of stimulation is kept constant the degree of this effect is proportional to the area of the C potential developed, i.e., to the number

TABLE 1

Threshold and maximal stimulating currents and conduction rates of the potential components of the vagus nerve of the turtle

EXPERIMENT	TEMPERATURE	COMPONENT												
		B_1			B_2			B_3			C		
		Threshold	Maximum	C.R. m.p.s.*	Threshold	Maximum	C.R. m.p.s.	Threshold	Maximum	C.R. m.p.s.	Threshold	Maximum	C.R. m.p.s.
1	22	1.5 C_2	4.5 C_2	10.0	7.5 C_2	12.0 C_2	4.9	45.0 C_2	90 C_2	1.4	67 C_2	67 C_4	0.6
2	22	1.5 C_2	6 C_2	9.6	7.5 C_2	22.5 C_2	4.8	24.0 C_2	45 C_2	1.1–0.8	90 C_2	45 C_4	0.6
3	23	1.5+C_2	4.5 C_2	11.9	9.0 C_2	22.5 C_2	4.6	16.5 C_2	67 C_2	1.2	22.5–67 C_2	135+C_2	0.5–0.3
4	23	1.4 C_2	4.5 C_2	11.0	6.0 C_2	9.0 C_2	4.8	22.5 C_2	67 C_2	1.4	45.0 C_2	135+C_2	0.4

Threshold values expressed in volts and condenser capacity, C_2 0.005 mf., C_4 0.1 mf.
* Conduction rates in meters per second.

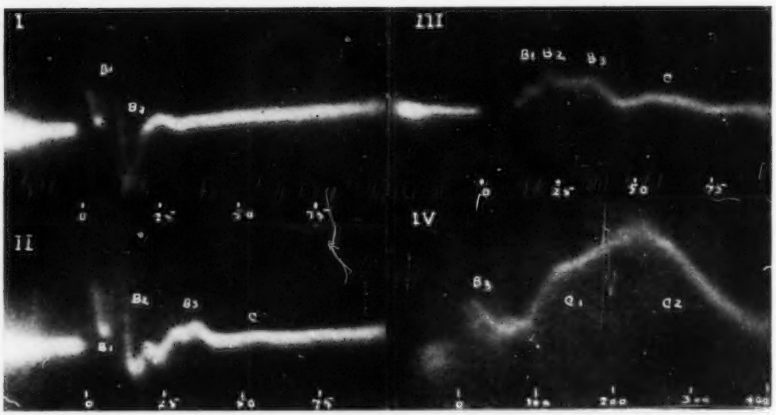

Fig. 1. Film I. Conducted action potential turtle vagus showing the B_1 and B_2 components. Stimulus 4.5 volts 0.001 mf. Conducting distance on the nerve 32 mm. Time in sigmas.

Film II. Conducted action potential same nerve, showing the B_1, B_2, B_3 and a trace of the C components, stimulus 16.5 volts 0.005 mf.

Film III. Conducted action potential same nerve showing the B_1, B_2, B_3 and a fairly well developed C_1 potential, stimulus 39 volts 0.005 mf.

Film IV. Conducted action potential same nerve showing the fully developed C_1 and C_2 potential components, stimulus 45 volts 0.1 mf. Conduction rate B_1 11.9 m.p.s. B_2 4.6 m.p.s. B_3 1.2 m.p.s. C_1 0.5 m.p.s. C_2 0.3 m.p.s.

of C fibers active. With the change in heart rate there occurs also an accentuation of the inotropic effect. The fully developed "C" potential will result in complete stoppage of the heart especially when the right vagus nerve is being stimulated. The increase in negative inotropic effect con-

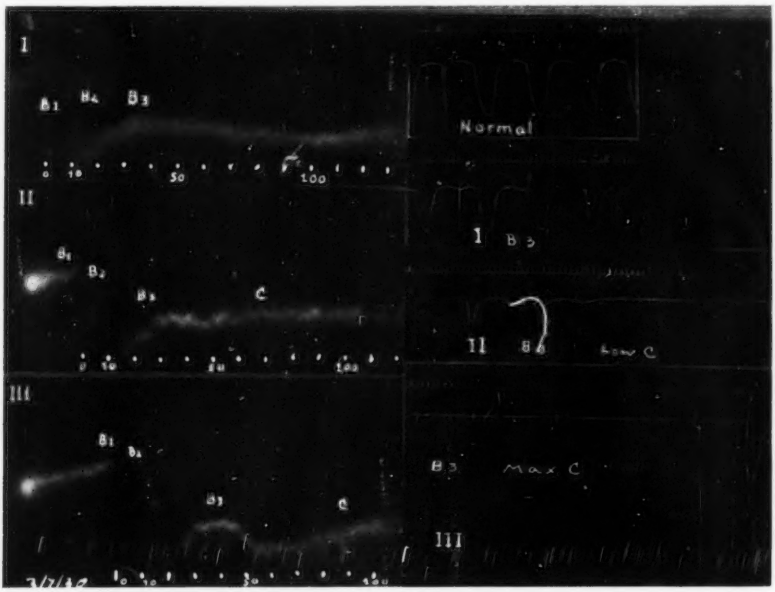

Fig. 2. Film I. Conducted action potential right vagus nerve of turtle showing the B_1, B_2 and a low B_3 potential. The atrial myogram marked I was taken at this point. This shows definite inotropism without any change in rate. Conduction distance on nerve 28 mm. Threshold B_1 3 volts 0.001 mf. B_2 15 volts 0.001 mf. B_3 35 volts, 0.001 mf. maximum 150 volts 0.001 mf. Threshold C_1 35 volts 0.001 mf. maximum 135 volts 0.1 mf.

Film II. Conducted action potential above nerve showing an increase in amplitude of the B_2 potential component and a low C potential component. The atrial myogram marked II was taken here. Note the marked inotropism and the marked change in heart rate, the usual finding on the right side.

Film III. Conducted action potential showing a maximum B_3 and C component. The atrial myogram marked III was taken here. It illustrates the effect of a single shock at this stimulus.

tinues throughout the range of the chronotropic effect. For reasons discussed below, one cannot escape the impression that while definite inotropic and chronotropic fiber groups are separable, the chronotropic response overflows or spreads secondarily to affect also the mechanism controlling the force of the heart beat. Both for the inotropic and the chronotropic

effects, increasing the frequency of stimulation up to a limit has an effect on the heart similar to that obtained by increasing the number of fibers stimulated. It is possible in certain preparations to completely stop the heart beat with a single maximual stimulus (fig. 2). Gilson (1930) has reported a similar finding. Similar experiments on the effect of vagus nerve stimulation in the bull frog yielded results similar to those obtained in the

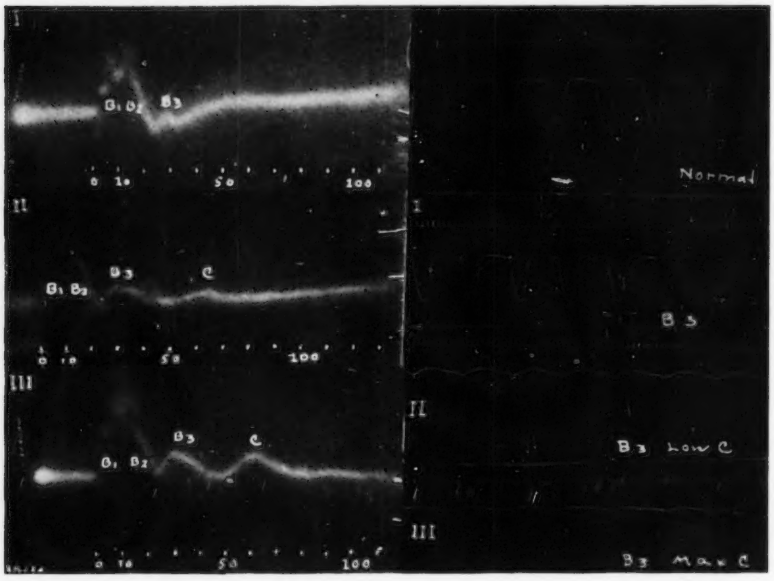

Fig. 3. Film I. Conducted action potential left vagus nerve showing B_1, B_2 and a low B_3 potential. Conducting distance on the nerve 27 mm. Atrial myogram I taken here. It shows the pure inotropic effect.

Film II. Conducted action potential above nerve showing B_1, B_2, B_3 and a low C potential. Atrial myogram II taken here. It shows a marked inotropic effect without any appreciable change in heart rate.

Film III. Conducted action potential above nerve showing the B_1, B_2, B_3 and a larger C potential. Atrial myogram III taken here. It shows an increased inotropic effect with slight but definite slowing of the heart rate.

turtle. It was possible to separate by threshold values a group of fibers whose effect was purely an inotropic one. Stronger stimulation always produced chronotropic as well as inotropic effects.

The experimental conditions for investigation of the effects of sympathetic fibers on the heart are less satisfactory for this type of study. The sympathetic branches to the heart in the turtle and frog are very short and

it is impossible to isolate them so as to permit the simultaneous recording of an electroneurogram and an atrial myogram. Threshold values may however be obtained. Our knowledge of the stimulus strength required for various fiber types in any particular animal is such as to permit reasonable assumptions as to the type of fiber being stimulated by a given current with a known stimulating circuit (Bishop and Heinbecker, loc. cit.). Since such assumptions are also consistent with the histological structure of the nerves concerned quite dependable conclusions may be drawn.

In bull-frog preparations yielding definitely positive inotropic and chronotropic changes as a result of sympathetic stimulation, it was only occasionally possible by nerve stimulation to obtain a threshold separation of any significant degree for the two effects as was obtainable in the vagus nerve studies (fig. 4). The threshold stimulus required was always adequate for the stimulation of unmyelinated fibers. At such a stimulus strength an increase in rate seemed generally to be anticipated by a few beats showing an increased contraction height. With stronger stimulation after a definite chronotropic effect it was possible to get a further increase in the inotropic effect without a corresponding increase in the chronotropic effect. In the course of subsidence of the sustained inotropic and chronotropic effects after cessation of stimulation it was not unusual for the chronotropic effect to fall off more rapidly than the inotropic. In certain preparations it was possible to diminish the chronotropic effect by rapid stimulation (200 or more per minute) with no appreciable lessening of the inotropic effect. This might have been due to a Wedensky-like inhibition in the fibers having the longer refractory period, or at the corresponding end organs. On slowing the stimulation rate the positive chronotropic effect would again increase. In other preparations where the auricular preparation was in a poorer state slowing of the rate of stimulation was not again followed by an increase in rate. Below this rate however inotropic and chronotropic changes occurred simultaneously.

Discussion. The separability of initial inotropic from the chronotropic effect in the turtle sinus and atrium on vagus stimulation is a finding which has been adequately supported by the experimental results of older investigators such as Heidenhain, (1882) and more recently by Gilson (1929). The association of specific potential components with primary inotropic and with the chronotropic effects has been made possible by the use of the cathode ray oscillograph as a recording mechanism, which because of its relative lack of inertia permits the conducted action potential of nerve to be seen as a complex of potential waves. The correlation of the fiber composition of a nerve trunk with its conducted potential form permits of deductions with regard to the fiber type responsible for the various potential components. It seems certain that the B potential in autonomic nerves is

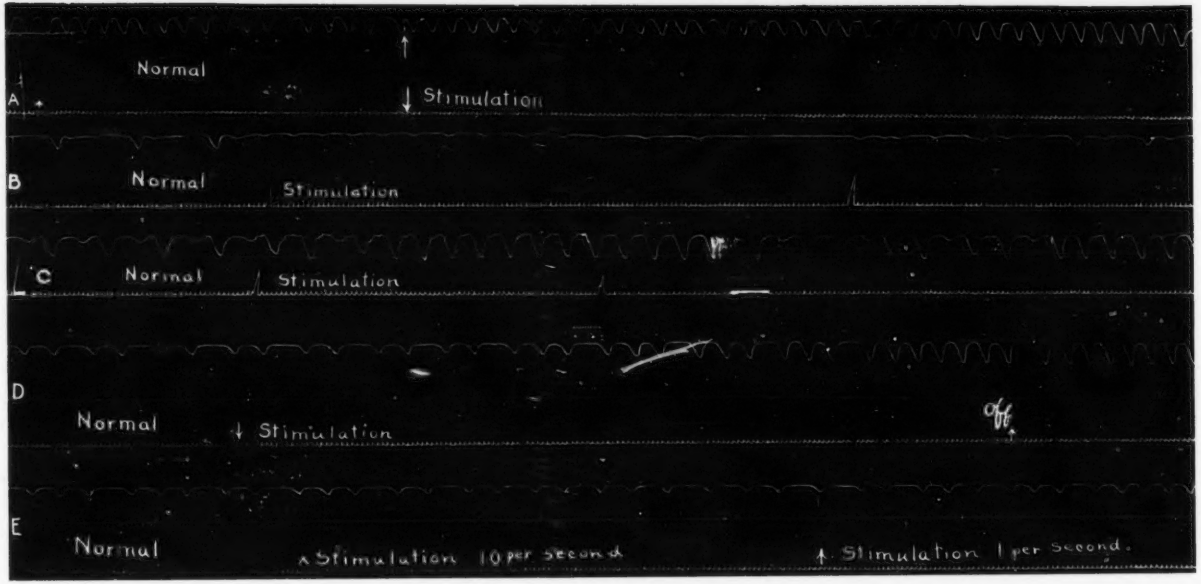

Fig. 4. A—Atrial myogram, bull frog, showing a purely inotropic effect on near threshold stimulation of cardic sympathetic branches. Time intervals $\frac{1}{5}$ second.

B—Atrial myogram, bull frog, showing a sino-atrial block accentuated by stimulation of unmyelinated vagus fibers.

C—Atrial myogram, bull frog, showing relief of sino-atrial block by stimulation of unmyelinated sympathetic fibers.

D—Atrial myogram, bull frog, showing inotropic and chronotropic effects from stimulation of unmyelinated sympathetic fibers. The inotropic effect precedes somewhat the chronotropic, rate and strength of stimulation constant throughout.

E—Atrial myogram, bull frog, showing a slightly negative rather than positive chronotropic effect on rapid stimulation (10 per sec.) of sympathetic fibers with no inotropic change. On slowing the rate of stimulation (1 per sec.) positive inotropic and chronotropic effects are obtained.

derived mainly from myelinated axons. Because the potential element designated B_3 in vagus and sympathetic nerves (Heinbecker, loc. cit.) seems more closely related in its properties to the general B potential than to the C potential it is here regarded as probably derived from myelinated axons. The C potential is known to be derived from unmyelinated axons (Heinbecker, 1929). Histological sections of the vagus nerve to the sinus and to the atrium reveal myelinated and unmyelinated axons of types identical with those characteristically efferent in other autonomic nerves (fig. 5). Beside these efferents, there are also myelinated axons of a type characteristically *afferent* elsewhere.

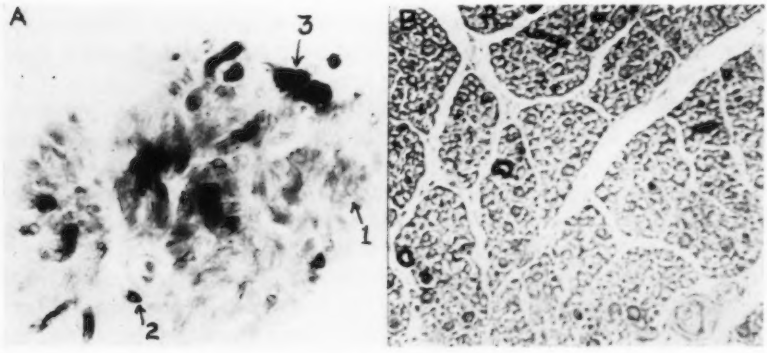

Fig. 5. A—Histological section turtle sino-atrial region showing three fiber types, unmyelinated, small thinly myelinated and a few larger and more thickly myelinated probably afferent in type, designated respectively 1, 2, and 3. We have secured no evidence to indicate that the small thinly myelinated are ever derived from the sympathetic supply to the heart. Osmic acid ×880.

B—Histological section monkey cardiac nerve. All fibers except a few, considered to be afferents are unmyelinated. This is typical of the sympathetic nerves to the heart of all animals so far studied. Osmic acid ×880.

In spite of overlapping of the two effects, it still seems not inconsistent with our experimental findings to consider that in the vagus nerve myelinated axons convey the impulses which primarily influence an "inotropic mechanism" in the sinus and atrium whereas unmyelinated axons innervate a "chronotropic mechanism." At the present time no attempt is being made to define this mechanism precisely. The expression of its function as studied in this paper is that of the rate of the sinus pacemaker. Obviously other of the vagus-innervated parts of the heart are likewise subject to effect. Sino-atrial conduction, etc., also begins to show depression within this stimulus range. It is certain that chronotropic effects are confined to the C group of fibers; but it is impossible to reduce the force of the beat to

zero by any increase in the rate of stimulation if the strength of stimulus is only sufficient to call forth a maximal B_3 wave. There are two obvious possibilities, either that the C group however distinct from the B_3 in its potential picture still contains inotropic fibers similar to the B_3 fibers, or that one given C fiber may give rise to both effects by itself, directly or secondarily, even though the B_3 fibers give rise to only one effect.

In the sympathetic nerves to the sinus and atrium the fibers initiating the inotropic and chronotropic effects are of one type, i.e., unmyelinated. This is demonstrated by histological study and it seems to explain our inability to often effectively separate, by threshold differences, fiber groups primarily inotropic or chronotropic in their influence. The positive inotropic and chronotropic effects both appear with shock strengths adequate to stimulate unmyelinated fibers in nerve trunks of the diameter here concerned.

The problem of determining the mode of peripheral action of the sympathetic fiber is quite similar to that in the case of the vagus. The evidence for a separate inotropic mechanism is here less convincing but by no means negligible. Certain preparations at near threshold stimulation values evidence a purely inotropic effect (fig. 4A). The recent results of Fischer (1930) are in agreement with this finding. The reason for the infrequence of such results on sympathetic stimulation as contrasted with the results on vagus stimulation probably rests in the fact that the fibers concerned are all of one type, i.e., unmyelinated. Consequently there could not be expected to be large differences in thresholds. The instances in which purely inotropic effects are obtainable may indicate that the larger unmyelinated fibers are the ones, in these preparations at least, to carry impulses producing such effects. The fact that a further increase in inotropic effect may be obtained after no further chronotropic change occurs also points to a separate inotropic mechanism. In a few preparations one may get a Wedensky-like inhibition of one effect (chronotropic) without an inhibition of the other (fig. 4E).

It seems advisable to point out that many of our experiments show a progressive increase of effect upon cessation of stimulation after one or several stimuli have been applied. Such effects are obtained when purely inotropic fibers as well as chronotropic fibers are stimulated. It is probable that a similar effect is exercised when the chronotropic mechanism indirectly influences the inotropic. It has been found here as well as in fibers to the eye (Bishop and Heinbecker, 1931) that a similar effect is produced by a high frequency and weak intensity of stimulation as by a low frequency and higher intensity; that is, that the total number of fiber responses is the significant factor. The summation effects can, in the light of our present knowledge, best be explained by the assumption that a peripheral substance is liberated whose action may increase for a time after nerve stimulation

and then decrease, possibly as the result of utilization or elimination of the active substance itself. Whether the effects here described are due to a direct action of the nerves stimulated or to an intermediate mechanism is as yet undemonstrated.

SUMMARY

In the turtle vagus nerve, fibers giving rise to separable potentials on conduction carry the impulses responsible for the negative inotropic and negative chronotropic effects in the sinus and atrium. These fiber groups have different thresholds and conduction rates. The fibers primarily responsible for inotropic changes are probably thinly myelinated, those responsible for chronotropic changes are unmyelinated.

In the bull frog sympathetic, unmyelinated fibers carry the impulses primarily responsible for both the positive inotropic and positive chronotropic changes in the sinus and atrium. They have a similar threshold.

The experimental findings are consistent with the interpretation that in the sinus and atrium the mechanisms responsible for the inotropic and chronotropic effects are influenced separately by fibers from the extrinsic nerves. The chronotropic mechanism within the sinus and atrium indirectly may also influence the inotropic mechanism.

This paper deals with one phase of a more extensive investigation of the functions of the autonomic nervous system being carried out by Dr. George H. Bishop and myself.

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STUDIES ON THE UTERUS

VI. The Effect of Oestrin on the Uterine Fistula During Pseudopregnancy[1]

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It has recently been shown in this laboratory that the quiescent uterus of a castrated rabbit doe exhibits marked oestral motility following the injection of minute quantities of Theelin[3] (Reynolds, 1931). Because of the profound effect exerted by this substance on the non-gravid uterus of the rabbit, and of its occurrence in large quantities in human urine of pregnancy, the question was raised, asking why abortion does not spontaneously occur since oestrin is present during pregnancy in both blood and urine in far greater amounts than at other times. Such a question presupposes that the action of oestrin on the human uterus simulates its action on the uterus of the rabbit. If indeed it should, that fact would be doubly striking, inasmuch as pregnancy in some of the lower animals may be terminated almost at will by the administration of oestrin, especially in the early stages of pregnancy. We were interested therefore, in this investigation, to determine the effect of the injection of Theelin on the motility of the uterus of the unanesthetized rabbit during early pseudopregnancy and later, while the uterus was under the influence of functional corpora lutea. In this way, one should be able to gain some information concerning the extent of mechanical factors in oestrin-abortion.

Experimental procedures and results. A description of the technic of making the uterine fistula and of recording therefrom has been given (Reynolds, 1930; Reynolds and Friedman, 1930a), and will not be repeated here, except as certain modifications were made in the course of the experiments.

[1] The expenses of this research have been largely defrayed by a grant to Prof. H. C. Bazett from the Committee for Problems of Research on Sex of the National Research Council. This fund was administered by Dr. M. H. Friedman.

[2] George Lieb Harrison Fellow in Medical Sciences, 1930–31.

[3] Theelin is the name of a crystalline oestrogenic substance obtained from human urine of pregnancy by the method of Veler, Thayer and Doisy, 1930, Journ. Biol. Chem., lxxxvii, 354. It is prepared and generously supplied to us by the Department of Experimental Medicine, Parke, Davis & Co.

We have already shown that marked uterine motility usually precedes coitus in the rabbit; that within the space of a few hours *post coitum* and preceding ovulation (10–12 hours *post coitum*) an abrupt cessation of this motility occurs, and that the uterus remains quiescent until the termination of a period of pseudopregnancy (15–20 days after sterile coitus; Reynolds and Friedman, 1930a). It is obvious that the onset of this effect precedes any possible influence of the corpus luteum, since it occurs before ovulation. We believed, therefore, that a marked difference in the threshold to Theelin might exist in the period just following coitus and later in pseudopregnancy, when functional corpora lutea were present. Such a difference might indicate either qualitative or profound quantitative differences in the inhibiting mechanism or mechanisms before and after the formation of corpora lutea.

With this in mind, we designed our experiments along two lines. First, Theelin was injected intravenously immediately after coitus, and records of uterine motility were obtained at intervals of a few hours and for some days thereafter; secondly, Theelin was injected in these and other rabbits four or more days after coitus.

Theelin immediately post coitum. Theelin was given immediately after the doe copulated, in four equal intravenous doses over a period of eight hours. Records of uterine motility were obtained prior to coitus and at intervals of 5 to 7 and 10 to 12 hours after coitus, and on subsequent days as indicated in table 1.

It will be seen that the initial phase of inactivity may be readily overcome with relatively small amounts of Theelin. We did not determine the minimal effective dose. In one instance, 40 r. u. per kilogram of body weight sufficed to abolish completely the usual quiescence for more than twenty-four hours. In all the remaining instances however, with amounts of Theelin varying from 5 to 20 r. u. per kilo, the inhibition became evident in five to seven hours, only to be definitely overcome in ten or more hours. A maximum of activity appeared twenty-four hours *post coitum*. This is the precise latency reported for the Theelin response in castrated rabbits (Reynolds, 1931). A definite Theelin response, therefore, was obtained. By forty-eight hours the uteri were nearly quiescent, and shortly thereafter full quiescence supervened and persisted, as indicated in table 1, and as described below.

Theelin after four days post coitum. The first group of rabbits used in this series of experiments were the five animals noted above. In three of these an amount of Theelin was injected on the fifth day of pseudopregnancy, equal to that which had previously been effective when injected on the first day. No response whatsoever was now obtained. In the remaining two does, over twice the maximum amount used in any of the first three instances was used. Again, no Theelin response was obtained. Clearly, a difference in threshold to Theelin between the first and fifth days of

pseudopregnancy was evident. It was decided, therefore, to inject two other does with excessive amounts subcutaneously, for it seemed possible that toxic effects might result from the administration of large quantities by the intravenous route. One doe was injected with a total of 2875 r. u. (1030 r. u. per kilo), during the third, fourth, fifth and sixth days of pseudopregnancy. On each day, six doses of Theelin were given at two hour intervals. The uterus of this rabbit showed no motility at any time. Similar results were obtained in another doe treated simultaneously. This doe

TABLE 1

Theelin administration on the first day of pseudopregnancy and the fifth. Intravenous injection immediately post coitum

Types of activity may be designated in the following manner:

0 = Quiescent condition of uterus in which no activity can be recorded with our system.

\+ = Feeble activity is that which with our system of recording gives records of contractions of ½ inch or slightly less.

++ = Moderate activity is that which varies between ½ inch to 2 inches, but which appears to average 1 to 1½ inches.

+++ = Marked activity is that which varies between 2 inches to 4 inches or more.

RABBIT	THEELIN R. U. KILO	MOTILITY ANTE COITUM	MOTILITY POST COITUM							
			5-7 hours	10-12 hours	24 hours	48 hours	3 days	5 days	Theelin r. u. kilo	6 days
1	20	++	+ Irregular	++ Irregular	+++	+ Irregular	+ Irregular	0	20	0
2	20	+++	+ Irregular	++	+++	0	0	0	20	0
3	40	++++	+++	+++	+++	++ Irregular	0	0	40	0
4	10	++	+ Irregular	++	+++	+ Irregular	0	0	80	0
5	5	++	0	++ Irregular	++	+ Irregular	0	0	100	0

received 2830 r. u. (1090 r. u. per kilo) on the sixth, seventh, eighth and ninth days of pseudopregnancy. At autopsy, these and all other does but one, to be specifically mentioned, showed entirely normal, healthy uteri. From these results, then, it became apparent that even these excessive quantities of Theelin were ineffective when given subcutaneously at this time. Accordingly, intravenous injections were again resorted to.

Another doe received, on the tenth day of pseudopregnancy a total of 800 r. u. of Theelin intravenously in seven doses given at two hour intervals, and 200 r. u. subcutaneously at night; on the eleventh day she received 600

r. u. intravenously, as before, and 400 r. u. subcutaneously at night. An average of 715 r. u. per kilo was thus given her in two days. The uterus of this doe remained quiescent till the thirteenth day, at which time bilateral ovariectomy was performed. A small amount of Theelin (12 r. u. per kilo in eight hours) was given on the fifteenth day after coitus, and a typical Theelin response was obtained. Thus, under favorable conditions (castration), this uterus was capable of a response. This experiment was repeated and confirmed on four other rabbits. Each received a total of 1600 r. u. of Theelin wholly by the intravenous route on the eighth and ninth days of pseudopregnancy. In no instance was a Theelin response elicited, despite the fact that a total of 600 to 730 r. u. per kilogram of body weight was injected in the several animals. Following castration on the eleventh day, however, three does showed a typical response to 15 r. u. per kilo, administered in the usual manner. The fourth doe was an exception only in that she spontaneously exhibited marked motility of the uterus after castration, against which a Theelin response cannot be demonstrated, for obvious reasons. From these data it is apparent that excessive amounts of Theelin, even given intravenously, are utterly without effect in eliciting any sign of motility from the third or fourth to the thirteenth days of pseudopregnancy.

In our previous work we have indicated that there is a considerable variability from animal to animal in the time at which motility returns, at the end of a period of pseudopregnancy (Reynolds and Friedman, 1930a). Despite this fact, we have been able to show in three experiments that it is unlikely that a hastening of this return to activity may be induced by Theelin injections at this time. One doe was injected with a total of 100 r. u. (50 r. u. per kilo) on the thirteenth day of pseudopregnancy. Her uterus remained quiescent until the sixteenth day, when it became feebly active, and on the seventeenth day marked activity was seen. No effect of Theelin was evident within forty-eight hours, when the effect in castrated rabbits injected with Theelin is nearly over. No sign of a response was obtained, therefore. A second doe which showed feeble activity on the seventeenth day received a total of 34 r. u. (10 r. u. per kilo) of Theelin. Although her uterus was already feebly active, it remained so for more than twenty-four hours, and it was not until forty-eight hours after the initial injection that a slight increase in motility was noted. A full return to marked activity occurred on the third day after the injections (twentieth day *post coitum*). Still a third rabbit whose uterus was quiescent on the eighteenth day, was injected with a total of 48 r. u. (20 r. u. per kilo). No response was seen in twenty-four hours. She was killed at this time, because the exposed uterine orifices were somewhat dry, due to occluded circulation resulting from the operative procedure. Autopsy revealed, however, that that part of the uterus which was beneath the ventral abdominal wall was entirely normal and healthy. We feel justified, therefore, in presenting the data

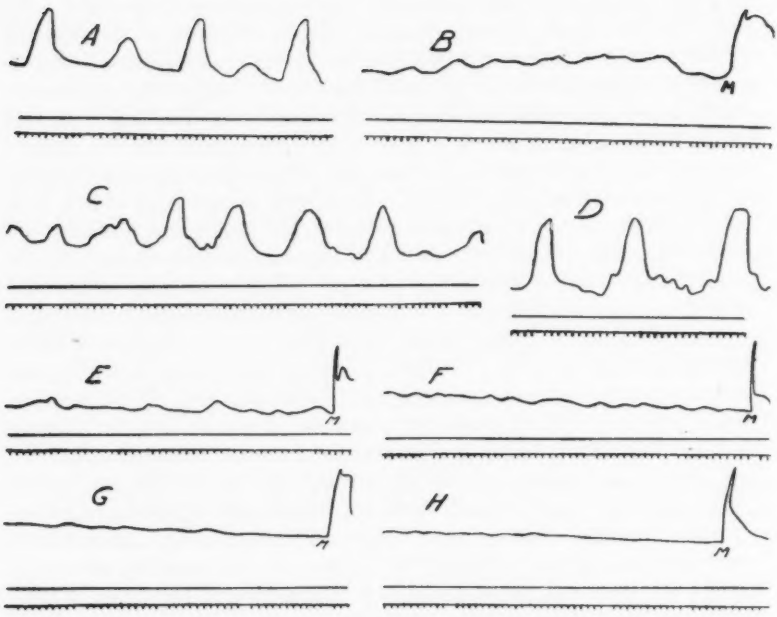

Fig. 1. Records from an experiment showing that the cessation of uterine motility following coitus in the rabbit may be overcome with oestrin (Theelin) when the injections of oestrin are commenced immediately *post coitum*, but that a similar amount administered on the fifth day is without effect on the motility of the uterus. Protocol: rabbit 2; litter, 3/10/31; fistula, 3/12/31; weight, 3.2 kilograms.

A 3/15 Normal motility. Coitus at 11:00 a.m. Theelin injections commenced immediately; four equal doses in eight hours. 20 r. u. per kilo; total 64 r. u. (1.28 cc., or 0.32 cc. per injection. 50 r. u. per cc.).

B 6:30 p. m.; 7½ hours *post coitum*.

C 10:00 p. m.; 11 hours *post coitum*.

D 3/16 24 hours *post coitum*. A normal Theelin response, with normal latency and of usual intensity (Reynolds, 1931). Uterus is normally quiescent at this stage, *post coitum*.

E 3/17 48 hours *post coitum*.

F 3/18 3 days *post coitum*.

G 3/20 5 days *post coitum*. Theelin again injected as above, 20 r. u. per kilo.

H 3/21 6 days *post coitum*. No Theelin response. Rabbit killed.

Autopsy: ovulation occurred. Eight new corpora lutea. Uteri normal and healthy, with no pus, inflammation or adhesions.

M—mechanical response, elicited by applying pressure to abdomen, to demonstrate that the balloon was compressible.

One-fourth original size.

from this animal, in support of that obtained from the other unquestionably acceptable ones. Accordingly, we may say that near the expected end of pseudopregnancy, the injection of Theelin in the amounts we have used has no observable effect in terminating the uterine quiescence that is an accompaniment of the condition of pseudopregnancy in the rabbit.

Discussion. The consistency of the results we have just described is striking, for not a single exception is to be noted in these experiments. So different is the threshold of the uterus to Theelin in the first few days of pseudopregnancy that one cannot but think that there may be at least two distinct factors concerned in initiating and maintaining the quiescent uterine state during pseudopregnancy. We have discussed these possibilities before (Reynolds and Friedman, 1930a; 1930b). From the present results, however, one fact is obvious; namely, that after the first few days of pseudopregnancy in the rabbit, an active inhibiting mechanism for the usual action of Theelin is present, *as regards uterine motility*. This is demonstrated beyond reasonable doubt, especially in those experiments where large amounts of Theelin were ineffective, and then, within the space of two days following castration these same uteri were susceptible to relatively small amounts of Theelin. This finding supplies the information with which we were chiefly concerned at the outset of this work, namely, that at a time when functional corpora lutea are present, oestrin is without effect in producing uterine motility. Parkes has argued (1929, p. 203) that it is difficult to attribute oestrin with the property of imparting activity to the uterus during oestrus, since it is found in great concentration during pregnancy, and so at a time when uterine motility would probably be unfavorable in the economy of the animal. These experiments appear to remove this difficulty, although it may yet be shown that oestrin during pregnancy may be more active in other respects than in its effect on uterine motility.

We must not lose sight of the fact, moreover, that our rabbits were not pregnant. It may so happen that conditions in the pregnant animal are different from what one encounters in pseudopregnancy, although no known humoral differences exist between early pregnancy and pseudopregnancy. Such a difference, however, might account for the reported cases of oestrin abortion, fully discussed by Parkes (1930; 1929, p. 118). The reports of oestrin abortion are conflicting and unsatisfactory, however. It would seem that no final clue as to the mechanism of this phenomenon may be had until one ascertains that abortion may or may not be induced in pregnant rabbits by the injection of Theelin in quantities less than, or equal to the ineffective amounts reported in this paper. If indeed it is found that abortion can be so produced, oestrin abortion may be due either to some toxicity of the oestrin on the pregnant animal, or to a more remote possibility, the depression of some other phase of endocrine function which is not affected in the pseudopregnant animal.

I wish to take the present opportunity, on completing this phase of my work, to acknowledge my indebtedness to Dr. Maurice H. Friedman. He is not only responsible for the initiation of these studies on the uterus, but has given me continued guidance and counsel and, not infrequently, help in the execution of the experiments themselves. He has also enabled me to visualize the possibilities that lie ahead in this field of endeavor.

SUMMARY

A difference in threshold of the uterus to the action of Theelin on uterine motility was found in the first few days of pseudopregnancy. The initial cessation of motility may be overcome with small amounts of Theelin. After four days this was found to be impossible with large amounts, whether given subcutaneously, intravenously, or by both methods over a period of two to four days. An acceleration in the return to activity following pseudopregnancy was not possible with the amounts employed. One may conclude that the effect of oestrin is definitely inhibited after the first few days of pseudopregnancy, as regards its action on uterine motility.

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STUDIES ON THE UTERUS

VII. Anaphylactic Response of the Non-Gravid Uterus of the Unanesthetized Rabbit[1]

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In general there is at present no satisfactory method of recording objectively the anaphylactic response in unanesthetized animals.

In most of the work done, the perfusion bath method, as described by Dale (1913), has been utilized in studying the response of uterine strips of the sensitized virgin guinea-pig uterus, and the response of other smooth muscle tissues excised from sensitized animals.

While this technique has yielded excellent results, it has many inherent shortcomings. In light of the investigations of Van Dyke and Hastings (1927), who showed that extremely small variations in physiologically essential ions, especially Ca, produced marked changes in the activity of the perfused uterine strip, it is obvious that ionic balance must be carefully controlled. Moreover, Hanzlik (1920) has demonstrated that the uterine strip response is not specific in all instances for the sensitizing antigen and may be obtained with a variety of unrelated substances.

Other workers using the smooth muscle response in anesthetized animals as an indication of anaphylactic shock, have obtained very varied results. Manwaring and Marino (1927), and Bally (1929) report that they obtained uniformly negative results on the smooth muscle reaction of urinary bladder in the anesthetized hypersensitive rabbit. These negative results may be due to the complicating factor of anesthesia since in the unanesthetized preparation urination during shock often occurs.

With the advent of the uterine fistula preparation described by Reynolds (1930a) an objective recording of anaphylactic shock in the unanesthetised animal was made possible.

Experimental procedure. The test animal employed is a female rabbit weighing approximately six pounds. Two series of experiments

[1] This problem was aided by a grant from the Committee for Research in Problems of Sex of the National Research Council. This fund was granted to Professor H. C. Bazett and administered by Dr. M. H. Friedman.

[2] George Lieb Harrison Fellow in Medical Sciences 1930–1931.

were performed. In the first series (table 1) sensitization was started immediately after parturition. In the second series of experiments, sensitization was started during pregnancy. In both series immediately post-partum the young were removed. Post-partum animals were used to insure good uterine motility (Reynolds, 1931).

Normal sterile horse serum without preservative (Mulford) was injected subcutaneously in four divided doses at four day intervals. The total sensitizing dose varied from 20 to 40 cc. A period of 14 to 25 days was allowed for incubation.

Three days before the end of the incubation period uterine fistulae were

TABLE 1

Series one—rabbits sensitized after parturition

Types of uterine activity may be designated in the following manner:

0 = Quiescent condition of uterus in which no activity can be recorded with our system.

\+ = Feeble activity is that which with our system of recording gives records of contractions of ¼ inch or slightly less.

++ = Moderate activity is that which varies between ¼ inch to 2 inches, but which appears to average 1 inch to 1½ inches.

+++ = Marked activity is that which varies between 2 inches to 4 inches or more.

NUMBER OF RABBITS	DEGREE OF UTERINE MOTILITY	SENSITIZING DOSE	SHOCK DOSE	RESPONSE OF UTERUS	REMARKS
		cc.	*cc.*		
2	++	20	1.0	Tetanus	Dyspnea
4	+++	20	0.3	Tetanus	No intra-abdominal pressure change
1	+++	20	0.3	Tetanus	Dyspnea, defecation, urination, death
1	++	30	0.25	Tetanus	Defecation
2	++	40	0.4–0.6	Tetanus	Dyspnea, defecation, death
1	+++	40	0.2	Tetanus	Urination, defecation

made by the method described by Reynolds (1930a). At the end of the incubation period the animal was prepared for recording uterine contractions. To determine whether or not the response was wholly uterine in origin, a small balloon was placed in the abdominal cavity through an incision made under local anesthesia (2 per cent novocain). The balloon was connected to a recording system similar to that used in recording uterine motility. This recorded changes in intra-abdominal pressure and in many experiments was so sensitive that intra-abdominal pressure variations synchronous with respiration were recorded. Changes in intra-abdominal pressure occurring with urination, defecation, dyspnea and in-

creased intestinal activity could always be recorded. That this procedure had no effect on the activity of the uterus was determined by recording before and after placing the balloon in the abdominal cavity.

At the beginning and end of each experiment the hand was placed on the abdomen and slight pressure was applied to demonstrate the free recording of intra-uterine and intra-abdominal pressure changes.

The shock dose of serum was injected into the marginal ear vein. The amount injected varied from 0.2 to 1.0 cc. To ascertain whether or not desensitization had been produced, an amount of serum equal to or greater than the initial shock dose was injected a short time afterwards. When no further response could be obtained with horse serum, 1.0 cc. of pituitrin was injected into the marginal ear vein.

Control experiments were made by injecting the unsensitized preparation with varying amounts of serum. Pituitrin was also injected into these rabbits.

After experimenting with various procedures, it was learned that the best manner in which to demonstrate a pure uterine response in anaphylactic shock is to use rabbits in which sensitization is started immediately after parturition. Following the proper series of injections (5.0 cc. each subcutaneously), at four day intervals, one may obtain, after an incubation period of twenty-one days, a regularly satisfactory response to a shock dose of 0.3 cc. serum.

RESULTS. 1. In the four unsensitized preparations used as controls, no change in uterine motility was recorded as a result of the intravenous injection of normal sterile horse serum. No signs of anaphylactic shock were observed. A characteristic response was obtained with the intravenous injection of 1.0 cc. pituitrin.

2. In eleven rabbits in which sensitization was started after parturition, the uterine motility was moderate or marked; and a definite response to the shock dose of serum was obtained (table 1). The chief characteristics of the response were:

a. The latent period of the uterine response varied between sixty and ninety seconds.

b. The uterine tetanus persisted for three to four minutes and was followed by a period of decreased uterine activity (less than the normal before shock) (fig. 1 *B*). In some preparations the response was purely uterine and free from abdominal pressure variations. In the remaining preparations of this series other signs of anaphylactic response could be observed. The other signs observed were polypnea, dyspnea, anemic appearance of the exposed portion of the uterine horns, increased gut motility as observed through the abdominal wall, contractions of the external anal sphincter, defecation yielding feces coated with mucus, marked cyanosis of gingival mucosa and in three cases death.

c. Within fifteen to twenty minutes uterine motility returned to the type seen before injection.

d. At various intervals up to one hour after the initial shock dose, desensitization of the preparation to serum was demonstrated in all the rabbits which did not succumb to the initial shock dose. Serum was not re-injected until uterine motility returned to pre-shock character. The number of injections necessary to produce complete desensitization varied, but in no instance was a second response elicited by the injection of a quantity of serum equal to the amount used to produce the initial shock (fig. 1 *C*). Two responses, an initial response, and a secondary response to a larger dose, were the greatest number obtained (fig. 1 *A* and *D*).

e. After desensitization, as indicated by a failure to respond to further injections of serum, a characteristic response was obtained with the intravenous injection of 1.0 cc. pituitrin in all the rabbits in this series. The latent period for this response was less than five seconds.

In the second series of six animals sensitization was started at various intervals after coitus. Two rabbits, in which the injections were started on the second and sixth days post coitum, did not drop litters. The other four rabbits dropped normal litters.

The four rabbits of the second series in which sensitization was started, up to twenty days post coitum, demonstrated feeble irregular uterine activity first prior to administration of the shock dose of serum, and in none of these preparations was there obtained any response, uterine or general, to the intravenous shock dose of serum, or to the injection of pituitrin.

The remaining two rabbits, in which sensitization was started eight days before parturition, did give a response to the intravenous injection of the usual shock dose of serum. One of these rabbits demonstrating feeble irregular uterine activity, did *not* show any detectable uterine response, although dyspnea, defecation and cyanosis were noted before death from anaphylactic shock. The other rabbit which had moderate irregular activity, showed a definite uterine response, as well as other signs of anaphylactic shock. After complete desensitization, a characteristic response to the intravenous injection of 1.0 cc. of pituitrin was obtained.

3. In a series of untreated rabbits in which motility was recorded in the same interval post-partum as in those preparations in series two, nine rabbits had marked regular uterine activity and two, moderate regular activity. This is in striking contrast to the series just described in which sensitization of the rabbits started during pregnancy.

Discussion. 1. The essential criteria, cited by Wells (1921), that define an anaphylactic response have been met in these experiments. The uterine response to the shock dose of horse serum depended upon the previous sensitization of the rabbit to that serum and the elapse of a suit-

able incubation period, inasmuch as *no response* was obtained with non-sensitized animals into which similar doses of serum were injected. After recovery from shock the uterus was found to be refractory to further in-

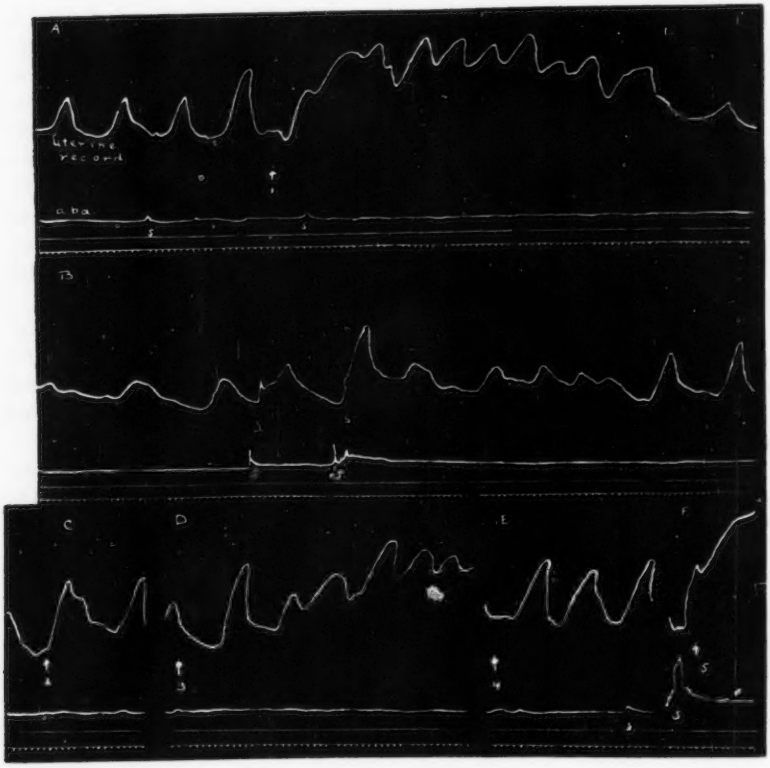

Fig. 1. Rabbit 5. Time intervals, 5 seconds. Sensitization was started after parturition with a sensitizing dose of 20.0 cc. horse serum and an incubation period of 21 days. Records *A* and *B* are continuous; 17 minutes elapse between records *B* and *C*; 5 minutes elapse between records *C* and *D*; 3 minutes elapse between records *D* and *E*; 8 minutes elapse between records *E* and *F*. *1*—inject 0.3 cc. serum intravenously; *2*—inject 0.3 cc. serum intravenously; *3*—inject 1.0 cc. serum intravenously; *4*—inject 1.0 cc. serum intravenously; *5*—inject 1.0 cc. pituitrin intravenously. *S*—slight struggle. (Reduced ⅓.)

jections of the same serum although it was capable of responding to a suitable stimulus as determined by the intravenous injection of pituitrin. The possibility that the uterine response may be caused by thrombosis or embolism of the pulmonary vessels is remote, since uterine responses were

obtained in several preparations in which all signs which would result from thrombosis or embolism were absent.

The marked difference in the latent periods of the uterine response to the shock dose of serum and to pituitrin is suggestive of the existence of some intermediate reaction which is necessary for eliciting a response with serum, since pituitrin probably acts directly on the muscle fibers (1913) and the latency in the reaction represents the time required for the circulation to transport the pituitrin in sufficient concentration to the uterus. This observation is at variance with the results of Dale (1909), who used the perfusion bath method and stated that the specific antigen, when introduced in a bath containing the muscle strip (guinea-pig uterus) produced a reaction immediately it came in contact with the muscle and quite as promptly as a preformed diffusible stimulant, such as pituitary extract. The significance of this marked difference in observed results is not as yet entirely clear.

The decrease in uterine activity (fig. 1, *B*) persisting for fifteen to twenty minutes after the initial tetanus is a constant finding and somewhat resembles, in appearance and duration, the decrease in activity following the response to pituitrin observed in these experiments and in those previously reported by Reynolds (1930b).

It may be noted that all the rabbits in which sensitization was started after parturition, demonstrated moderate or marked uterine activity at the time of recording (table 1). Hammond and Marshall (1925) have shown that if the young be immediately removed after parturition, the female rabbit comes into heat. Reynolds and Friedman (1930) demonstrated that female rabbits prior to coitus usually exhibit moderate or marked uterine activity. It is apparent that repeated subcutaneous injection of serum at a time when the female rabbit is in "heat" does not alter the uterine motility which is characteristic of oestrus.

2. A different situation exists, however, in those instances in which the series of injections was started during pregnancy. The spontaneous motility following parturition was regularly found to be feeble, with one exception. This is in *marked* contrast to the series of controls of eleven non-sensitized rabbits, nine of which showed marked and two moderate activity at a similar period post-partum. It may be concluded that the subcutaneous injection of serum during *early* pregnancy alters the state of the rabbit so that after parturition and removal of the young, uterine motility is markedly decreased.

A peculiar observation was made in that four rabbits sensitized soon after coitus did not show any signs of a uterine or general response to the injection of the shock dose of serum. Two rabbits, in which sensitization was started later in pregnancy, eight days before parturition, did give an anaphylactic response to the shock dose of serum. Since similar sensitizing

doses and incubation periods were used as in series one (I), the objection cannot be raised that conditions necessary for sensitization were not present in these rabbits. The number of experiments in this series is small. Nevertheless, in comparison with the constant production of hypersensitivity in series one (I), it is suggestive that some as yet unexplained relationship exists between the occurrence of sensitization and the course of pregnancy. What factor or factors are concerned must await further investigation.

The four rabbits in which there was feeble irregular activity did not respond to the intravenous injection of pituitrin. This confirms the previous work of Reynolds (1930b) who showed that the response to pituitrin in the non-gravid uterus depends upon the degree of spontaneous uterine activity existing prior to the injection of that drug.

SUMMARY

1. The uterine fistula preparation affords an adequate means whereby anaphylactic shock may be recorded in the unanesthetised rabbit.

2. Rabbits sensitised *after* parturition retain the normal type of uterine activity and respond with a marked uterine tetanus in anaphylactic shock.

3. Rabbits sensitised early in pregnancy have not, in our experience, shown normal post-partum uterine activity.

4. Our data suggest that sensitisation during early pregnancy is not easily accomplished.

5. A marked difference exists in the latent period of the uterine response to pituitrin and to the shock dose of serum in the sensitised animal. The significance of this has been briefly discussed.

We wish to take this opportunity to acknowledge our indebtedness to H. K. Mulford Co. for their generous coöperation in supplying the horse serum used in these experiments.

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STUDIES ON THE CORONARY CIRCULATION

I. Absorption of Lactic Acid by the Heart Muscle[1,2]

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Accumulating information on the formation of lactic acid in the tissues and its diffusion into the blood has shown the necessity for additional data bearing on its distribution by the blood and on the factors governing its disposal. In an earlier communication (McGinty, 1929) it was demonstrated that brain tissue normally removes lactic acid from the blood and that during impaired oxidations in the brain, brought about experimentally, lactic acid is added to the blood during its circulation through the brain capillaries. It was concluded that this process was reversible and that a state of equilibrium exists between lactic acid absorption and outward diffusion and that it plays a part in the acid base balance of brain tissue. That an exchange of lactic acid between blood and tissues is likewise concerned with the buffering system of the blood, was suggested by Macleod and Knapp (1918). Subsequent work by a number of investigators has lent support to their views (Anrep and Cannan, 1923; Long, 1924; Eggleton and Evans, 1930).

Himwich and his collaborators (1928) and Cori and Cori (1928) showed that under certain conditions, lactic acid produced in skeletal muscle passes into the blood, is carried to the liver and stored as glycogen to be liberated subsequently as glucose. The latter investigators emphasized the importance of lactic acid transfer in the carbohydrate balance of the body. In connection with his experiments on absorption of lactic acid by the liver, Himwich (1928) in a few experiments obtained results which indicated that the heart absorbed lactic acid from the blood. In view of the importance of these findings it seemed desirable to continue an investigation of this phase of lactic acid disposal in tissue, and to learn whether the rate of removal is influenced by changes in cardiac activity, alterations in the coronary circulation and by disturbances of oxidations in cardiac

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muscle. These were induced by stimulation of the vagus and of the stellate ganglion and by the intravenous injection of "pitressin."[3] As an index to lactic acid exchange between coronary blood and heart muscle, analyses were made on arterial blood and the venous blood from the coronary sinus.

METHOD. Dogs were anesthetized either with urethane, Gréhant's mixture of chloroform and alcohol or with sodium amytal. Small amounts of morphine preceded the general anesthetic. The thorax was opened in the midline during constant artificial respiration. After injection of heparin, a glass cannula of a size and shape so as to cause least interference with venous outflow into the coronary sinus was placed in the sinus near its junction with the right auricle A ligature which served to secure the cannula, effectively closed the opening into the auricle. The rubber tubing leading from the cannula was led over the chest wall to a level slightly below the heart in order to place the sinus contents under a very small negative pressure imitating normal conditions in the thorax. Volume flow was measured in most of the experiments by collecting the blood in a graduated cylinder and signalling at 25 cc. volumes. This coronary blood and additional defibrinated blood from a second dog in amounts equal to the hemorrhage of sampling was returned to a femoral vein at as uniform a rate as possible. Arterial blood pressure, heart rate, coronary sinus volume flow and pulmonary ventilation were recorded on a kymograph.

Arterial blood samples from a T cannula in one carotid artery and coronary venous samples drawn into a syringe inserted through the rubber tubing near the end of the coronary sinus cannula were withdrawn at approximately the same time. Lactic acid analyses (Friedemann, Cotonio and Shaffer, 1927) were made on filtrates prepared according to the Folin-Wu procedure in most experiments and according to the second macro technique of Somogyi (1930) in the remainder. Both filtrates give identical lactic acid results on the same blood over all ranges of concentration of lactic acid. Absorption of lactic acid by the heart is expressed in two ways, absorption per 100 cubic centimeters of coronary sinus blood flow and absorption per minute, calculated from the arterial venous difference in milligrams per 100 cc. blood and coronary volume flow in cubic centimeters per minute. Since part of the blood entering the coronary arteries is returned to the heart chambers by way of the foramina Thebesii, these results apply only to that portion of the cardiac muscle whose venous drainage is into the coronary sinus. However, since Evans and Starling (1913) and others have called attention to the fact that coronary sinus venous flow bears a constant ratio to the entire coronary flow under a wide variety of cardiac activities, the results obtained are strictly comparable.

RESULTS. In 226 out of 230 pairs of blood samples from 16 dogs under a variety of experimental conditions, coronary venous blood contained less

[3] Vasopressin, Parke, Davis & Company.

lactic acid than arterial blood by differences ranging from within the analytical error of 3 mgm. per cent to 23 mgm. per cent. If a comparison be made between the height of the lactic acid level of arterial blood and the degree of absorption as shown by the difference between arterial and coronary venous concentrations, it becomes evident that there is no constant relationship between the two factors. Arterial venous differences under a given set of conditions were no greater toward the end of a series of experiments when the arterial concentration of lactic acid was high than at the beginning of the experiment when it was low. Arterial lactic acid content ranged from 25 to 130 mgm. per cent.

Thirteen observations of the effect of faradic stimulation of the vagus on lactic acid absorption were made on 9 dogs. The vagus was stimulated shortly following the initial arterial and coronary venous blood samples for periods of 55 to 200 seconds, the current being adjusted so as to slow the heart considerably but without causing complete arrest. Blood samples were drawn near the end of the period of stimulation and in from one to five minutes following stimulation.

Details of three representative experiments of this series are given below. In the first observation, with the fall in blood pressure from 84 to 60 mm. Hg and of the heart rate from 173 to 70 beats per minute during the 90 second period of stimulation, coronary sinus volume flow decreased from 112 cc. to 30 cc. per minute, 27 per cent of the initial volume flow. An initial arterial venous difference of 5.5 mgm. (arterial, 40.6, venous, 35.1 mgm.) increased to 8.3 mgm. (arterial, 38.6, venous, 30.3 mgm.) per 100 cc. of blood. Despite the increased absorption of lactic acid from each unit of circulating blood, the gradient of movement of lactic acid from blood to muscle decreased as shown by a fall in absorption of lactic acid per minute from 6.1 to 2.5 mgm. One minute after the end of the period of vagal stimulation, a partial return to the initial state was indicated by an arterial venous difference of 7.6 mgm. per cent and minute absorption of 6.0 mgm. Although coronary volume flow remained at the somewhat low level of 80 cc., considerable recovery was evident.

In another experiment coronary sinus volume flow decreased from 75 to 40 cc. per minute with the fall in blood pressure and heart rate. The arterial venous difference increased from 4.8 to 7.0 mgm. per cent and minute absorption decreased from 3.6 to 2.8 mgm., the results resembling those of the first observation. In a one minute period following stimulation, recovery was less evident than in the first experiment since minute absorption remained low at 2.65 mgm. despite the considerable improvement in flow of blood through the heart. These two observations were characteristic of the usual results obtained. In 3 of the 13 vagal experiments, the effects of stimulation were at variance in certain particulars. An illustration of this type showed that during the period of stimulation

the coronary volume flow fell from 80 to 47 cc. per minute, 59 per cent of the pre-stimulation flow. In contrast to the first two experiments in which there occurred an increase in the difference between the arterial and coronary concentrations of lactic acid, the arterial venous difference was decreased from 9.1 to 7.4 mgm. per cent during 60 seconds of stimulation and continued to fall to 5.7 mgm. per cent one minute following stimulation when the coronary flow had returned nearly to its pre-stimulation rate. As in the first two experiments, minute absorption, however, decreased from 7.3 to 3.5 mgm. and to 3.3 mgm. one minute after stimulation. Complete recovery was later demonstrated

A résumé of all observations during stimulation of the vagus is shown in table 1. In 8 experiments, there was recorded a decrease in arterial blood pressure, heart rate and in volume flow of coronary blood. The fall in volume flow amounted to 27–62 per cent (av. 53 per cent) of the initial flow before stimulation. In 2 experiments blood pressure and heart rate fell, and since according to Anrep and Segall (1926) stimulation of the vagus causes a constriction of the coronary vessels, it is reasonable to assume that coronary volume flow decreased in these observations. In all 10 of these experiments an increase in absorption of lactic acid per unit flow of blood was observed. Average absorption of 6.1 mgm. per 100 cc. of blood before stimulation increased to 10.4 mgm. per 100 cc. of blood during stimulation. The increase was moderately uniform and well beyond the possibility of errors of analysis.

In 3 experiments, blood pressure, heart rate and coronary volume flow decreased, the average fall in blood flow amounting to 58 per cent of the initial flow. Contrary to the results of the first series, arterial venous differences fell from an average of 8.5 mgm. before stimulation to 6.6 mgm. per 100 cc. of blood during the period of stimulation.

In all observations there occurred a uniform decrease in the calculated absorption of lactic acid per minute. The constancy of the results obtained with stimulation of the vagus makes it highly probable that similar results would have appeared if the coronary sinus volume flow had been recorded in the 2 experiments in which this measurement is lacking. The average decrease in minute absorption in 11 experiments was 2.8 mgm. lactic acid.

The effects of faradic stimulation of the stellate ganglion were followed in 13 observations on 8 dogs. Ordinarily the left stellate was stimulated usually with intact vagi, for periods of 60 to 180 seconds and with a current strength sufficient to bring about a definite increase in heart rate and strength of cardiac beat. Blood samples were taken near the end of the period of stimulation and in from one to five minutes following stimulation. Although the results were not as uniform in certain respects as during stimulation of the vagus, one fairly representative observation is given in detail below. In this experiment coronary sinus volume flow increased

TABLE 1

Resumé of all observations on absorption of lactic acid by heart muscle during variations in coronary sinus volume flow of blood and of cardiac activity

NUMBER OF EXPERIMENTS	EFFECT ON BLOOD PRESSURE	EFFECT ON HEART RATE	EFFECT ON CORONARY SINUS VOLUME FLOW	EFFECT ON ARTERIAL-VENOUS DIFFERENCE	EFFECT ON LACTIC ACID ABSORPTION
Effect of stimulation of vagus (9 dogs) Average duration of stimulation, 2 minutes					
				mgm. per 100 cc.	*mgm. per minute*
8	Decrease	Decrease	Decrease	Increase	Decrease
2	Decrease	Decrease		Increase	
3	Decrease	Decrease	Decrease	Decrease	Decrease
Total 10	Decrease	Decrease	27–62% (av. 53%) of initial (8 expts.)	6.1 to 10.4 (av. 10 expts.)	6.0 to 3.2 (av. 11 expts.)
3	Decrease	Decrease	54–62% (av. 58%) of initial	8.5 to 6.6	
Effect of stimulation of stellate ganglion (8 dogs) Average duration of stimulation, 2 minutes					
5	Increase	Increase	Increase	Decrease	Decrease
3	Increase	Increase	Increase	Decrease	Increase
1	Increase	Increase	Increase	Decrease	No change
3	Increase	Increase		Decrease	
1	Increase	Increase	Increase	Slight increase	Increase
Total 13	Increase	Increase	115–235% (av. 145%) of initial (av. 10 expts.)	7.7 to 4.1 (av. 12 expts.)	2.9 to 2.2 (av. 10 expts.)
Effect of injection of pitressin (10 dogs) Average amount, 2 cc. (20 units)					
7		Decrease	Decrease	Decrease (outward diffusion, 2 expts.)	Decrease (outward diffusion, 2 expts.)
3		Decrease		Decrease (outward diffusion, 2 expts.)	
4		Decrease	Moderate decrease	Moderate increase	Slight increase
Total 10		Decrease	12–95% (av. 45%) of initial (7 expts.)	8.4 to 2.5 (av. 6 expts.)	5.8 to 2.3 (av. 5 expts.)
				8.3 to −3.4 (av. 4 expts.)	5.2 to −1.2 (av. 2 expts.)
4		Decrease	68–95% (av. 78%) of initial	5.1 to 7.9	3.0 to 3.9

from 118 to 132 cc. per minute. The initial arterial venous difference of 7.5 mgm. decreased to 1.6 mgm. per 100 cc. of blood. Minute absorption decreased from 8.8 to 2.1 mgm. Two minutes following stimulation blood pressure and heart rate had fallen nearly to the initial level. Coronary volume flow decreased to 71 cc. per minute. The arterial venous difference increased to 6.2 mgm. per cent and minute absorption to 4.4 mgm., showing a definite tendency toward recovery

In table 1 is a résumé of all observations made during stimulation of the stellate ganglion. In all experiments there appeared constantly an increase in blood pressure and heart rate. Coronary volume flow was recorded in 10 of the 13 observations. The changes in flow varied from 115 to 235 per cent of the initial flow. In the three experiments in which blood flow was not recorded it is believed that coronary volume flow increased as in the recorded observations since the blood pressure and heart rate rose during the period of stimulation. Anrep and Segall (1926) have demonstrated, furthermore, that stimulation of the stellate ganglion causes coronary dilatation. In every experiment but one, absorption per unit flow of coronary blood decreased. The average arterial venous difference before stimulation in 12 observations was 7.7 mgm. per 100 cc. of blood. During stimulation the average arterial venous difference fell to 4.1 mgm. per cent. In a single experiment the arterial venous difference increased from 3.2 to 4.5 mgm. per cent.

Minute absorption decreased in 5 experiments, increased in 4 experiments and showed no change in one. The average change in all experiments was from 2.9 to 2.2 mgm. absorption per minute.

Since it is known that pitressin diminishes the heart rate and exerts a powerful constricting effect on the coronary vessels when present in the blood in high concentrations (Clark, 1929), the effects of this hormone on disturbances in lactic acid absorption were studied. One to 2.5 cc. equivalent to 10 to 25 units was injected rapidly into the femoral vein. Blood samples were withdrawn in from one to two minutes after injection or at such time when the slowing of the heart was maximal and in from 3 to 6 minutes after injection. The results of such observations were followed in 14 experiments on 10 dogs. The details of 3 representative experiments are given below. In the first of these three observations, coronary volume flow was reduced from 101 to 61 cc. per minute, 60 per cent of the pre-injection flow two minutes after the administration of 1 cc. of pitressin. The arterial venous difference fell from 5.5 mgm. to 1.8 mgm. per 100 cc. of blood. Minute absorption decreased from 5.6 to 1.1 mgm. Five minutes after injection, coronary flow had continued very low at 45 cc. but the arterial venous difference rose to 4.8 mgm. per cent and minute absorption of lactic acid increased to 2.1 mgm.

In another experiment after administration of 10 units of pitressin,

coronary volume flow decreased to 12 per cent of the initial flow, the arterial venous difference decreased by 2.0 mgm., from 7.5 to 5.5 mgm. per cent and minute absorption fell from 5.4 to 0.5 mgm. In a period of 5 to 6 minutes after the injection volume flow had improved, although still very low, and the previous absorption of lactic acid had given way to an outward flow of 4.1 mgm. per 100 cc. of blood. By calculation the outward movement of lactic acid from muscle to blood amounted to 1.3 mgm. per minute. The conditions existing previous to administration of pitressin were later reëstablished. The above experiments are illustrative of the results of a group of 10 of the 14 experiments. Another experiment illustrates the results obtained in 4 observations.

In contrast to the considerable reduction in coronary volume flow as shown in the two preceding observations, the reduction in this case was much less. Instead of the well defined decrease in arterial venous difference and decreased minute absorption seen in the former experiments, there occurred a rise in the difference in concentration of lactic acid between the arterial and coronary venous blood, as well as a slightly increased absorption per minute. These effects persisted and were evident 5 minutes after the injection.

In table 1 is a résumé of the 14 pitressin observations on 10 dogs. The average amount injected was 20 units (2 cc.). The effects on arterial blood pressure are not listed since they were irregular and depended on the relative effects of peripheral vascular constriction and on lowering of the heart rate.

In 7 experiments the coronary volume flow fell by 12 to 92 per cent of the initial, an average of 45 per cent. The arterial venous difference decreased in 5 of these experiments and showed increased amounts of lactic acid in venous over arterial blood in 2 experiments. Minute absorption decreased in 5 experiments of this group of 7 and gave way to an outward movement of lactic acid from muscle to blood in 2 experiments.

In 3 experiments, coronary volume flow was not recorded. The arterial venous difference fell in 1 of the 3 experiments and decreased to the point of outward diffusion in 2. Minute absorption or outward diffusion could not be calculated because of the lack of data on coronary volume flow.

Of all 10 experiments the average coronary volume flow during the observed height of the pitressin effect amounted to 45 per cent of the pre-injection flow. The average arterial venous difference in 6 of the 10 experiments before injection was 8.4 mgm. per cent. After injection, these differences fell to an average of 2.5 mgm. per cent, a decrease of 5.9 mgm. per 100 cc. of blood flowing through the coronary vessels. In 4 experiments of this group, an average inward movement of lactic acid of 8.3 mgm. per 100 cc. blood flow changed to an average outward movement of 3.4 mgm. per 100 cc. Calculated from volume flow records and arterial

venous differences, minute absorption fell by 3.5 mgm. in 5 experiments and reversed to a minute outward flow of lactic acid of 1.2 mgm. in 2 experiments.

The mean arterial venous difference increased by 2.8 mgm. per 100 cc. blood in 4 of the 14 observations. In contrast to those 10 experiments in which there was observed a decrease in arterial venous difference, the coronary sinus volume flow of blood in these experiments was but moderately decreased. Absorption of lactic acid per minute was scarcely effected, increasing by only 0.9 mgm.

A recapitulation of all experiments shows that stimulation of the vagus caused a uniform decrease in coronary volume flow, a decided increase in the arterial venous difference in 10 of 13 observations and a uniform and definite decrease in minute absorption. Stimulation of the stellate ganglion caused constantly increased coronary volume flow, a decided decrease in arterial venous difference in 12 of 13 observations but an uncertain change in minute absorption of lactic acid. The mean variation indicates a slight reduction in absorption. With injections of pitressin in which the effects on the circulation through the heart were small as shown by moderate decreases in volume flow, the arterial venous differences rose slightly. Minute absorption likewise increased by a small and probably insignificant amount. With more effective doses as shown by considerable decreases in coronary volume flow arterial venous differences decreased. In 4 such experiments the decrease gave way to an outward flow of lactic acid as indicated by the higher concentration in venous blood over that of arterial blood. Minute absorption fell in a like manner and to the point of outward diffusion in 4 experiments.

Discussion. The results of these experiments make it probable that the heart absorbs lactic acid from the blood in the coronary circulation under normal conditions as well as under the circumstances described. The lowest initial arterial lactic acid level observed in the series of experiments was 25 mgm. per cent. The objection may be raised that at this concentration absorption might readily take place. But absorption of lactic acid by the heart muscle at much higher concentrations does not yield average arterial venous differences any greater than arterial venous differences at arterial concentrations nearer the normal. Hence it is likely that absorption occurs in the normal animal. In the 6 experiments on the heart reported by Himwich in which there was a uniformly high (from 41 to 131 mgm. per cent) concentration of lactic acid in the arterial blood, there is no evidence that absorption by the heart muscle increased as the arterial concentration rose.

Whether lactic acid passes from arterial blood into heart muscle or from muscle to venous blood depends largely on the concentrations in the two tissues. The concentration of lactic acid in muscle may be regarded as

dependent on the state of equilibrium between its production and its disposal which in turn is dependent on the state of oxidations. With the gradient of movement of lactic acid remaining the same, absorption by the heart muscle per unit flow of blood must be inversely proportional to the velocity of the coronary circulation. That this is indeed the case, has been demonstrated in the experiments on vagal and stellate stimulation, and following the minimally effective administrations of pitressin. In all of these there were but relatively moderate changes in coronary sinus volume flow of blood and the rate of absorption calculated on the basis of unit volume flow of blood showed almost uniformly an inverse relationship to the rate of flow.

During vagal stimulation, coronary volume flow not only decreases as a result of the lowered blood pressure and heart rate and because of the coronary vascular constriction, but there is in addition a certain degree of oxygen want as a result of the diminished blood supply. Increased production of lactic acid follows and the gradient of inward diffusion is lowered as is evident from the uniformly decreased minute absorption. On the other hand, with the retardation of the flow of blood through the coronary system, the movement of lactic acid to muscle from any given volume of blood is facilitated.

During stimulation of the stellate ganglion the results on minute absorption of lactic acid show virtually no change. The increased cardiac activity from stellate stimulation may possibly have caused increased lactic acid production in the muscle but was probably compensated for by the increased volume flow of blood through the cardiac vessels. Here again, the inverse relationship between velocity of coronary blood flow and arterial venous difference is illustrated.

Following administration of pitressin there resulted a greater or lesser constriction of the coronary vessels with a subsequent proportionate fall in coronary volume flow. At the same time the heart rate decreased, due in part to the decreased blood supply and the resulting disturbance in oxidations and in part to a direct or reflex effect on the muscle (Clark, 1929).

With the lesser effective injections of pitressin during which a relatively small decrease in volume flow was recorded, minute absorption changed but little. The average of four experiments in which coronary volume flow fell to only 78 per cent of the pre-injection flow, showed a slightly increased minute absorption. A possible explanation of this may be ascribed to the relatively greater decrease in cardiac activity over the contemporary oxygen supply. A decrease in lactic acid production would increase the inward gradient of diffusion.

Following the more effective injections of pitressin, coronary volume flow fell to the point where a considerable degree of oxygen want must have

existed in the cardiac muscle. Lactic acid formation increased beyond its rate of removal. As evident by the lowered minute absorption, the inward gradient was definitely decreased in six of the ten observations of this group of experiments. In the other four, lactic acid production so greatly exceeded its rate of removal as to cause an outward gradient from muscle to blood.

SUMMARY AND CONCLUSIONS

To study the factors involved in the formation, distribution and disposal of lactic acid in the body, analyses were made of simultaneous samples of arterial and of coronary venous blood of the beating heart in situ. Arterial blood pressure, heart rate and, in most experiments, respiration and coronary volume flow measurements were recorded.

In 226 of a total of 230 pairs of samples in 16 dogs under a variety of experimental conditions, coronary venous blood contained less lactic acid than the corresponding sample of arterial blood.

Faradic stimulation of the vagus in 13 observations on 9 dogs for periods of about two minutes caused diminished blood pressure, heart rate and coronary sinus volume flow (recorded in 11 experiments) during the period of stimulation. There occurred a corresponding decrease in minute absorption of lactic acid from an average of 6.0 mgm. to 3.2 mgm. per minute. The reduced gradient of inward movement of lactic acid from blood to muscle is explained by increased production of lactic acid in the muscle because of impaired oxidations induced by the fall in coronary volume flow of blood. Despite the lowered gradient, there was observed an increased difference in lactic acid content of arterial and venous blood. This difference bears an inverse relationship to the velocity of flow.

Faradic stimulation of the stellate ganglion for periods of about two minutes in 13 observations on 8 dogs was followed by increased blood pressure, heart rate and coronary volume flow (recorded in 10 experiments) during the period of stimulation. A corresponding and uniform decrease in absorption per unit volume flow of blood was observed. Minute absorption decreased in five experiments, increased in four and remained the same in one. An average of all experiments indicated virtually no change in minute absorption.

Moderately effective injections of pitressin caused diminished heart rate, a moderate fall in coronary volume flow and slightly increased minute absorption. Arterial venous differences increased slightly.

More effective injections of pitressin, with considerable reductions in coronary volume flow resulted in greatly decreased minute absorption, due to the reduced supply of oxygen to the cardiac muscle. In four experiments the falling gradient of lactic acid absorption gave way to an outward flow of lactic acid from muscle to blood.

The heart normally absorbs lactic acid from the blood. This absorption is independent apparently of the level of concentration in arterial blood and continues to obtain even during considerable reductions in coronary volume flow of blood, although the rate of absorption is diminished. With extensive impairment of oxidations lactic acid may form to such an extent in cardiac muscle as to diffuse into the blood.

The author wishes to express his gratitude to Dr. George Bachmann for his constant interest in the advancement of the problem.

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THE RELATION OF PITRESSIN TO WATER INTERCHANGE IN FROGS

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The effect of pituitrin and pitressin on the water content of muscular tissues has been the subject of recent publications by Glass (1), Clark (2), and Parhon, Marza and Cahane (3). The significant idea brought out by each paper in its own way, was that extracts of pituitrin have a tendency to cause an accumulation of water in striated muscle tissue of mammals. The question arose as to whether similar results could be obtained with the muscles of frogs, whose environment is entirely different. This question was the starting point of a series of experiments on the gastrocnemius muscle, both isolated and intact. The investigation of this problem later gave rise to a related set of experiments concerning the effect of pitressin on the water content of the whole frog. In this connection, work was also done on frogs in which the cloaca was tied off, and later, on skinless frogs. The results obtained indicate that injections of pitressin cause considerable quantities of water to be absorbed and retained by the whole frog as well as by the muscle tissue. Evidence will be presented that these effects are due, for the most part, to an increased permeability of the skin.

METHOD AND RESULTS. The first part of the problem, the effect of pitressin on the water content of the isolated muscle of the frog, was attacked in the following manner. The gastrocnemius muscles were removed from the body, and, after being attached to previously weighed muscle hooks, were weighed at 15 minute intervals. In order to do this, they were suspended from one arm of an analytical balance. The right gastrocnemius was submerged in Ringer's solution of known concentration, and served as a control, while the left gastrocnemius, the experimental, was submerged in a Ringer's solution of the same concentration, but with the addition of 0.3 cc. of pitressin. In all weighings uniform care was taken to avoid any pronounced error due to any excess amount of saline which might be adhering to the surface of the muscle. Three different concentrations of Ringer's solution were used: normal, hypotonic, and hypertonic, the hypotonic being 25 per cent less than isotonicity, and the hypertonic 25 per cent above isotonicity.

The results obtained in these experiments are summarized in figure 1. The broken line represents the experimental muscle and the solid line, the control muscle. As the curves indicate, there is no appreciable difference in weight changes between the control muscle and the one exposed to pitressin; this would indicate that pitressin in the doses used has no effect on the water absorbing power of the isolated muscle.

Similar experiments were likewise performed in which surgical pituitrin and pitocin were used, but the results were in no way markedly different from those just presented. These effects on the isolated muscle of the frog have been previously reported (4).

Since pitressin had no appreciable influence on the isolated muscle, experiments were then undertaken to discover its effect on the muscles taken from the body after pitressin had been injected in the whole animal. In such experiments some type of control was obviously necessary. This was accomplished by removing the right gastrocnemius before the injection and the left after the injection of pitressin. The technique of carrying out this experiment was as follows: four normal sized frogs (Rana pipiens) were placed in tap water at room temperature for one hour before using to avoid any variations in weight due to environmental changes. The right gastrocnemius was then removed by placing a tight ligature around the thigh muscles, so as to avoid bleeding; the leg was then cut off below the ligature and the gastrocnemius isolated and weighed on an analytical balance as previously described. Then three of the four frogs were injected with 0.3 cc. of pitressin, and the fourth was kept as a control. After an hour and a half all the frogs were killed and the left gastrocnemii were removed and weighed. The percentage increase in the control may be found in table 1, group I, column I, while the percentage increase of the three injected frogs may be found in column II. The results of a group of similar experiments are in groups II and III, with the exception that the frogs were allowed to remain injected for time periods of three and six hours respectively before the removal of the left gastrocnemius.

As the table indicates, there is no appreciable increase in weight in the control frogs from group to group, whereas the experimental muscles show a pronounced increase, the greatest weight change manifesting itself within three hours after the injection. In a comparison of this sort, it is necessary that the normal weights of the right and left gastrocnemii be nearly identical. In order to verify this, the comparison was made between the weights of the right and left gastrocnemii of ten normal frogs. The results showed that the percentage difference in eight cases was less than 1 per cent. In two cases the variation in weight of the right and left gastrocnemii was between 1 and 3 per cent. These latter changes may possibly account for some of the variations in table 1.

Along with these experiments, work was carried on to find the effect

of pitressin on the weight changes of the whole frog. The technique used in following these variations was to weigh the whole frog in a previously weighed covered beaker. A beam balance accurate to 0.1 gram was used. In all the weighings the beaker was previously rinsed with water, and any accumulation of water coming into the beaker with the frog was drained out before weighing. The experimental frogs were then injected in the dorsal lymph sac with doses from 0.3 to 0.5 cc. of pitressin, and returned to the water. Admittedly, this dose may be rather large compared with that of other workers, but in no case did the frogs manifest any ill effects from the dose administered. Weighings were taken at intervals of about one hour for a period of six to eight hours. This type of weighing probably cannot attain a high degree of accuracy due to the fact that somewhat different

TABLE 1

A comparison in weight changes in gastrocnemius muscles of frogs when the whole frog is previously injected with 0.3 cc. of pitressin

Percentage increase in weight in muscles due to pitressin

GROUP I (INJECTED 1½ HOURS)				GROUP II (INJECTED 3 HOURS)				GROUP III (INJECTED 6 HOURS)			
Control		Experimental		Control		Experimental		Control		Experimental	
Number	Per cent increase	Number	Per cent increase	Number	Per cent increase	Number	Per cent increase	Number	Per cent increase	Number	Per cent increase
		1	27.09			1	18.32			1	0.55
1	2.89	2	16.39	1	1.45	2	7.56	1	1.04	2	17.52
		3	22.31			3	12.44			3	8.96
		1	15.13			1	17.55			1	2.43
2	0.92	2	19.60	2	3.26	2	11.28	2	8.12	2	2.44
		3	17.74			3	11.08			3	12.90

amounts of water adhere to the frog's skin at different weighings. However, the differences between experimental and control frogs were far outside any experimental error.

The curves of figure 2 are plotted from the data obtained in these experiments and represent an average of four frogs. The upper curve shows an increase in body weight of over 14 per cent due to pitressin which reaches its maximal point in five hours, while the lower curve shows no marked change occurring in a normal uninfected frog which was used as a control with the same environment.

These results clearly indicate that pitressin has a pronounced influence on the body weight of the intact frog. This increase in weight is assumed to be due to the accumulation of water in the tissues. A few experiments in which the muscles of pitressin injected frogs were dried to constant weight

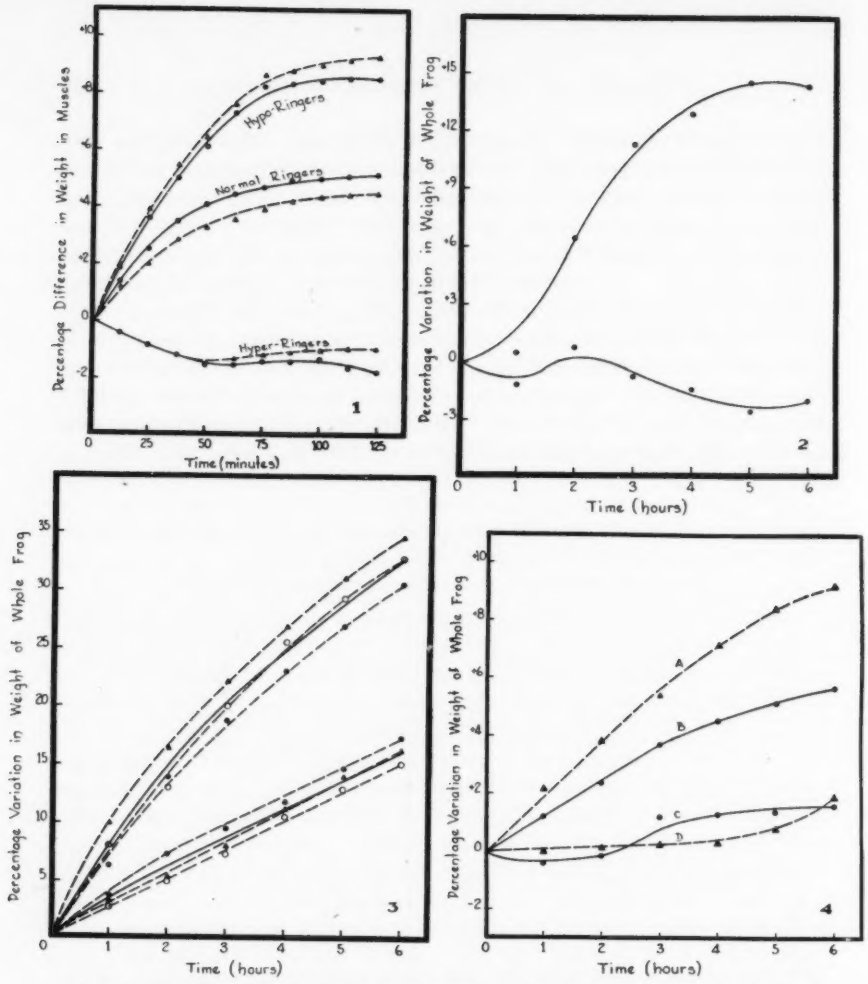

Fig. 1. The effects of pitressin on the water absorbing power of the isolated muscle. The right and left gastrocnemius muscles were submerged in 25 cc. of different strength Ringer's solution. The right gastrocnemius served as a control, while the left was the experimental. Three cubic centimeters pitressin was added to the solution in which the experimental muscles were submerged. The solid line represents the average weight changes of five control muscles, while the broken line shows the effects on the experimental muscles.

Fig. 2. An average curve representing weight changes in four frogs after an injection of 0.5 cc. pitressin into the dorsal lymph sac.

Fig. 3. A comparison in weight changes in frogs with the cloaca tied. Each of the upper curves represents the average percentage weight changes of three frogs injected with 0.3 cc. pitressin on three different days. The lower curves represent the weight changes in the control frogs into which no pitressin was injected.

Fig. 4. A comparison in weight changes in normal and skinless frogs when submerged in normal Ringer's solution. Curves *A* and *B* represent weight changes in four normal intact frogs. The broken line *A* shows the effect of injections of 0.3 cc. pitressin, while the solid line *B* serves as the control. Curves *C* and *D* represent weight changes on skinless frogs. Curve *D* represents injected frogs.

at 100°C. indicated this fact. This change might be due either to the stimulation of, or the injury to, some normal body function. The two most probable organs affected would seem to be the kidneys, which regulate water loss, and the frog's skin, which controls in a major part the intake of water under normal conditions.

A large quantity of research has been done concerning the effects of extracts of pituitrin on the mammalian kidney, and it has been shown by Fee (5), as well as other investigators (6), (7), (8), that pituitrin has a marked antidiuretic effect. Therefor, it seemed necessary to investigate the effect of pitressin on water retention by the kidney. Although no experiments were performed directly on the kidney in this case, information was obtained indirectly by tying off the cloaca and thereby cutting off the main avenue of water loss. Both control and experimental frogs were prepared in this way, the experimental frog always being injected with pitressin. They were then submerged in tap water for six hours, and weighings were taken at hour intervals with the same technique as previously described.

Figure 3 represents the average results obtained by performing the same experiments on three different days; as the curves show, the results, obtained on different days with different frogs, are practically identical. The lower curves picture the increase in weight produced in the normal frogs; a constant increase is evident with a gain of 15 per cent over normal body weight within the six hours. The upper curves represent the average changes produced in frogs injected with pitressin. There occurs a pronounced increase in body weight above the control frogs. Upon examination of the curve, it will be noticed that the percentage increase of the pitressin frogs above the control frogs is slightly above 15 per cent. This percentage increase is practically the same as that obtained in curves II where the injected frogs showed an increase of approximately 15 per cent over the normal. The solid line representing an average of the three, shows that the rate of increase in the injected frogs is greater in the first four hours and tends to decrease from there on, probably because the effects of the pitressin are wearing off, whereas, in the control, the increase is constant.

If the pitressin, in producing weight increases, had done so by decreasing kidney function, there would have been no difference in weight between the experimental and controls, since the cloaca was tied and the urine elimination in both frogs arrested. But since the results show that frogs in this condition, when pitressin was injected, took up an excess of water nearly comparable to that found in former experiments, it may be assumed that the pitressin was acting on the organism in some other manner than on the kidneys, or avenue of water loss. Experiments will now be presented to show that the skin very likely is involved in this large intake of water under the influence of pitressin.

This stage of the problem was attacked by placing normal and skinless frogs in normal Ringer's solutions, to determine whether pitressin acted primarily on the skin or muscular tissues. The technique used in skinning the frogs was that described by Adolph (9). The reason for submerging the frogs in Ringer's solution instead of water was to prevent the pronounced osmotic effects in skinless frogs when placed in hypotonic solutions. Weighings were made as before, at hour intervals over a period of six hours.

The results are presented in figure 4, where *A* and *B* show the changes produced in the intact frogs' weight, and curves *C* and *D* represent the skinless frogs. *A* and *D* represent the experimental or injected frogs, and *B* and *C* the controls. In the case of the intact frogs the curves show an appreciable difference between the control and pitressin frogs; but no such divergence is evident in the skinless frogs, whose weight undergoes only slight alterations. Curve *B* verifies the results reported by Adolph, who found that a normal frog when placed in Ringer's solution increases considerably in weight, probably due to some alteration of the skin. Curve *A* shows that intact frogs under the same environmental conditions, with the addition of a pitressin injection, swell to an even greater extent. This difference between the control and experimental frogs shows that the substitution of Ringer's solution for tap water has no inhibitory action upon the swelling power of the frogs injected with pitressin. The remarkable feature of this set of curves, however, is the fact that neither the control skinless frogs (curve *C*) nor the injected skinless frogs (curve *D*) show any appreciable increase in weight, nor any marked divergence, one from the other, indicating that in skinless frogs this pitressin effect is absent.

Discussion. It has been found in all these experiments that pitressin has little or no effect upon the isolated muscle; also that the antidiuretic action of pitressin is not responsible, in any measure, for the results, since they occur with the cloaca tied; but the comparison of effects of pitressin on intact and skinless frogs has yielded definite evidence that the skin plays an important rôle in the absorption of water in pitressin injected frogs. The probability is that pitressin in some way causes an alteration in skin permeability. As to the exact nature of the action, no direct evidence can be offered. Adolph (9) has recently reported that permeability of the skin can be altered by single pithing, anesthetics, and salt solutions, both hypotonic and hypertonic. Perhaps the pitressin acts in a similar way on the skin, or it may be that it acts directly on the skin through the circulatory system; it is also possible that increased permeability may be related to the change in melanophores, which Hogben and Slone (10) have found to be definitely affected by extracts of pituitrin. Although it is impossible at this time to give an exact explanation for the manner in which pitressin acts on the frog's skin, the results nevertheless clearly manifest effects pronounced to warrant further investigation.

SUMMARY

1. Pitressin has no effect on the water absorbing power of the frog's isolated gastrocnemius muscle.

2. Pitressin, when injected into the whole frog, causes a 14 to 15 per cent increase in weight within 3 to 4 hours, with the effects disappearing after about 6 hours.

3. Gastrocnemius muscles removed from previously injected frogs show an increase in weight over the control of about the same order as those weight changes in the whole frog.

4. The antidiuretic effects of pitressin do not play a major part in weight changes found in these experiments.

5. It appears that the skin is primarily responsible for these weight increases due to pitressin injections.

6. These results indicate that an alteration in skin permeability might be responsible for these changes.

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THE SITE OF FORMATION OF BILIRUBIN

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Since the publication of Mann, Sheard, et al. (1) concerning the site of formation of bilirubin, it has been the general opinion in this country that these authors have definitely settled the long disputed problem. According to these investigators, in dogs nearly all of the bilirubin is produced by the bone marrow, while the liver and the spleen add only insignificant amounts to the circulating blood. Such conclusions are, however, contradictory to previous experimental evidence presented by others, which indicates the liver as the main site of bilirubin formation.

As an example of these experiments, I wish to quote only the work of Gilbert, Chabrol and Benard (2). They studied in a series of fourteen dogs, the increased elimination of bilirubin in the bile following injection of hemoglobin by means of a fistula of the common bile duct and ligature of the cystic duct. While an almost immediate and considerable increase of bilirubin was readily demonstrated in the bile, at no time was bile pigment found in the blood even when, during the height of elimination, the total blood of an animal was examined after concentration.

The evidence presented by Mann, et al. is based on spectro-photometric measurements carried out on extracts of blood serum derived from different sources in the dog. It is the purpose of this paper to demonstrate that these measurements should not be considered as sufficient evidence to practically exclude the liver as the site of bilirubin formation and to ascribe this function to the bone marrow. This will be shown by a comparative study of some of the spectrophotometric curves and the presentation of original spectrophotometric findings.

The spectrophotometric readings are plotted in curves. The abscissa represents the wave lengths of the light while the ordinates indicate the light transmission in per cent. Curves showing more light absorption were assumed as indicative of higher bilirubin concentration. From these curves, Mann et al. concluded that there is more bilirubin in venous blood than in arterial, that there is more bilirubin in the veins returning from the bone marrow than in other veins. Such conclusions would be valid if there were sufficient reason to believe that all of the observed light absorp-

tion was due to the presence of bilirubin and that no other pigments were interfering. Such proofs have not been presented. In a later paper Sheard, Mann and Bollmann (3) present spectrophotometric data obtained from solutions of bile and of purified bilirubin. By establishing curves corresponding to different known concentrations the authors show that the curves closely follow the Beer-Lambert laws which postulate that the concentrations are proportional to the natural logarithm of light transmission. If Mann et al. in their previous experiments had dealt with pure enough solutions of bilirubin, the same laws would hold true by comparing these latter curves with those of purified bilirubin. Such a comparative analysis shows that the curves derived from purified bilirubin solutions have a sharper rise than those relating to a serum extract. Figure 1 shows curves from pure bilirubin solutions and such from serum extracts. (These

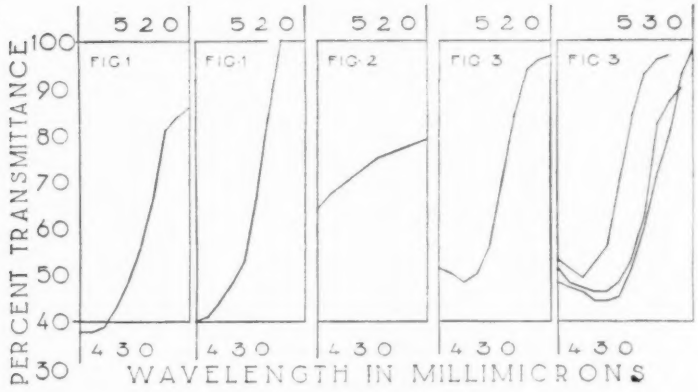

Fig. 1

curves are taken from the paper about the sites of bilirubin formation (1) and the studies of purified bilirubin (3).) The typical difference between these two groups of curves can readily be demonstrated by the application of the Beer-Lambert laws. Following the measurements of Sheard, et al. and my own observations, these laws are strictly valid for solutions of purified bilirubin or bile. If, therefore, the measurements with serum extracts represent quantitative evaluations of bilirubin they must be comparable with the ones obtained from bilirubin solutions.

Table 1 depicts such a comparison. The last column represents the quotient $\frac{\log J_1}{\log J_2}$ which, following the Beer-Lambert laws, must be constant. It is noted that the value of this quotient rises steadily with higher wave lengths. Therefore, the Beer-Lambert laws which, as have been shown

by Sheard, et al. and my own observations, can be applied to bilirubin solutions, are void in regard to these curves. It therefore appears most likely that the serum underlying the curve contained at least one other pigment, besides bilirubin. It is possible to predict that such an additional pigment has a spectrophotometric curve which rises slower than bilirubin with increasing wave length and consequently would be somewhat similar in color to bilirubin, but more yellowish than red.

I have searched for such a pigment in dogs and I believe to have found it by extracting dog fat with acetone and alcohol after careful elimination of hemoglobin. By adding such extracts to solutions of bilirubin it is possible to duplicate the same type of deviation from the bilirubin curve as found in the serum curves of Mann. It should be remembered that bone marrow may contain 90 per cent fat (4). Figure 2 shows the spectro-

TABLE 1

WAVE LENGTHS	PER CENT TRANSMITTANCE = J_1 SERUM FROM SPLENIC VEIN (1)	$-\log J_1$	PER CENT TRANSMITTANCE = J_2 BILIRUBIN SOLUTION (2)	$-\log J_2$	$\frac{\log J_1}{\log J_2}$
430	38	0.420	40	0.398	1.05
440	38	0.420	41	0.381	1.08
450	39	0.409	44	0.357	1.14
460	43	0.367	48	0.319	1.15
470	49	0.310	53	0.276	1.17
480	56	0.252	67	0.174	1.45
490	67	0.174	84	0.076	2.29
500	81	0.092	96	0.018	5.05

1. Cf. Reference sub 1, figure 4, curve 1.
2. Cf. Reference sub 3, figure 1 A, curve 6.

photometric qualities of this lipochrom. Its curve complies with the prediction.

Table 2 represents some application of the Beer-Lambert laws to my own findings. All my measurements were made with the photoelectric spectrophotometer from the American Photo-electric Corporation in New York. The adjustment of the wave length scale was controlled with a helium lamp. The first curve shows the behavior of a solution of bilirubin (5) in acetone and alcohol. The other one is based on a mixture of bilirubin and the lipochrom. The quotient of the logarithms in the last column shows the same typical behavior in the other chart representing a comparison between a serum extract and a bilirubin solution.

Figure 3 shows on the left side a curve from a bilirubin solution (5), on the right side three curves of solutions having the same concentration of bilirubin but containing different amounts of lipochrom. Their appear-

ance is as similar to the bilirubin curves as the serum curves of Mann et al. are to their bilirubin curves, the type of deviation as analyzed in the charts being essentially the same. It is furthermore illustrated that in these curves higher light absorption does not necessarily mean higher bilirubin concentration, as all of the three curves have the same bilirubin concentration. That the pigment extracted from fat is different from bilirubin, is evident by its curve.

Consequently, the spectrophotometric curves published by Mann, et al. do not constitute a basis for quantitative comparisons of bilirubin in dog serum extracts and therefore the conclusions concerning bilirubin concentrations, drawn from these spectrophotometric measurements, lack proof.

TABLE 2

WAVE LENGTHS	PER CENT TRANSMITTANCE = J_2 BILIRUBIN SOLUTION	$-\log J_2$	PER CENT TRANSMITTANCE = J_1 SOLUTION OF BILIRUBIN + LIPOCHROM	$-\log J_1$	$\frac{\log J_1}{\log J_2}$
430	51	0.293	51	0.293	1.00
440	50	0.301	48	0.319	1.06
450	48	0.319	47	0.337	1.06
460	50	0.301	46	0.328	1.09
470	56	0.252	46	0.337	1.34
480	70	0.155	48	0.319	2.06
490	84	0.075	53	0.276	3.68
500	94	0.027	62	0.208	7.70

SUMMARY

1. The spectrophotometric curves published by Mann, et al. in order to show the sites of bilirubin formation are essentially different from spectrophotometric controls based on bilirubin solutions.

2. This difference is apparently due to the presence of another pigment.

3. A pigment which, when added to bilirubin solutions, changes the spectrophotometric curves in a way to make them similar to the serum curves, can be extracted from dog fat.

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THE EFFECT OF DIFFERENT PER CENTS OF PROTEIN IN THE DIET

VII. Life Span and Cause of Death[1]

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Five groups of albino rats, designated I, II, III, IV and V, were fed continuously throughout life and succeeding generations on carefully prepared synthetic diets containing 10, 14, 18, 22 and 26 per cent of protein respectively. The plan and procedure of the experiment have already been given (Slonaker, 1931a). This paper deals with the results of the original 18 pairs in each group and with such remates as were necessitated on account of the premature death of one or both of the first matings. Their offspring and succeeding generations will be dealt with in other papers.

It is practically impossible to make lifelong experiments and observations in regard to diet on human beings because of the lack of control of the food intake for a lifetime and the environmental conditions of the subjects while the diets are modified in known and definite ways. The diet and the environment can, however, be completely controlled in experiments on the rat. The chemistry and physiology of nutrition in man and the rat are sufficiently similar that the establishment of a nutritional principle by experiment with one of them may in all probability be expected to apply to the other species as well. Mitchell (1929) concludes that with proteins, as with other nutrients, successful nutrition and continued health and physiological efficiency are possible over a wide range of intake. The body is on continual guard against the potentially deleterious action of its intermediate digestion products, bacterial end products, and intermediate metabolites. He deems it unwise that a diet should contain too little or too much of any food.

That growth is influenced by the source of the protein in the diet has been shown by Hoagland and Snider (1927). Using a diet containing 10 per cent of protein they found that better growth occurred when the protein in the

[1] This research has been assisted by The Department of Physiology, the National Research Council through its Committee for Research in Problems of Sex, the National Live Stock and Meat Board, the Fleishmann Company and their successors, Standard Brands, Inc., and the coöperation of Albers Bros. Milling Company.

diet was derived from animal sources, such as beef, lamb and pork, than when derived from wheat, oatmeal, navy beans and other plant sources. They also found that almost as good results obtained when 50 per cent of the protein was from animal sources and 50 per cent from plant sources. They conclude "since the protein of beef, as well as many other animal products, greatly enhances the value of the protein of grains, it is highly probable that the cereal proteins will be utilized very efficiently when they are included in a mixed diet containing meat and other animal proteins." We have shown in this experiment (Slonaker, 1931a) that best growth both in maximal weight and body length was obtained on a mixed diet containing approximately 14 per cent of protein and that the lowest maximal weight was in the group receiving 26 per cent protein. We further showed (Slonaker, 1931d) that when the loss of activity due to reproduction was taken into consideration, group II on 14 per cent protein registered the greatest number of miles run and was followed in a descending order by groups III, I, IV and V. This again indicates that a diet containing slightly in excess of 14 per cent of protein produced the greatest spontaneous muscular efficiency in the rat. The average daily energy intake of food up to approximately 150 days of age from greatest to least was in the order of I, II, III, IV and V, but the average per cent increase in body weight during this time from greatest to least was in the reverse order (Slonaker, 1931c). After deducting the energy used in activity and lost in the feces during the first 150 days the amount available for growth and basal metabolism from greatest to least was in the order of I, II, III, IV and V, but the total protein content of this available energy was 570, 673, 742, 845 and 969 calories respectively. This showed that up to 150 days of age there was a close correlation between percentage increase in body weight and the amount of protein in the diet. For the whole life span the average daily food intake in grams from greatest to least was group III, 20.11; V, 19.94; I, 18.36; II, 17.92; and IV, 16.00.

The males in general showed a greater mortality than the females in each group. Sterility was more prevalent in the males of each group. The order of fertility in both sexes from highest to lowest was groups II, I, III, IV and V. The per cents of fertile males in group II was 87 and in group V, 33. In these same groups the fertile females were 100 and 50 per cent respectively (Slonaker, 1931b). Since each of the diets contained the same ingredients and since the increasing per cents of protein in the different groups from 10 per cent in group I to 26 per cent in group V were obtained wholly by a proper addition of meat scrap to the diet of group I one is led to conclude that either the meat scrap contained something which tended to produce sterility, or that protein per se in per cents above or below an optimum of approximately 14 per cent tended to inhibit reproduction both in causing sterility and in reduction of the size of the average

litter born. The length of the reproductive span in both sexes from longest to shortest was groups II, I, III, IV and V. The shortening of the reproductive span was due more to a delayed beginning of reproduction rather than to an earlier secession of this activity. The average number of litters, the average size of the litters and the average number of young born per pair from greatest to least were in the order of groups II, I, III, IV and V. In general the weight of the offspring (generation I) at birth tended to increase as the protein content of the diet became greater. The per cent of daily gain in body weight of the young from birth to the weaning age in general increased from group I to group V with each addition of protein in the diet. The differences in birth weight and growth were at least partly due to the varying size of the litters in the groups. The mortality of the young during the nursing period was least in group II and greatest in group V. In general the females showed a greater mortality than the males of the same group during lactation. Group II reared a larger per cent of the young born than any of the other groups (Slonaker, 1931e). The loss of weight of the mothers while nursing was greatest in group I and became less as the per cent of protein in the diet increased (Slonaker, 1931f). Since the young made the best gain during the nursing period and the mothers lost the least on the rich protein diet it is evident that for the best good of both young and mother while nursing the diet should contain at least 26 per cent of protein.

Such significant factors as heredity, environment and nutrition play an important part in determining the length of the life span of an animal. All our rats were from the same stock, and all the groups were subjected to the same environment. Any variation, therefore, in the life span of the five groups can be attributed to the differences in nutrition. Certain ingredients in the diet are essential for normal life and physiological activities. Glasser (1923) has shown that the house fly lives but a short time (1 to 8 days) and lays no eggs when fed exclusively on protein or products of protein hydrolysis. Similar results obtained when the diet consisted of raw starch. Sucrose lengthened the life but did not induce egg laying. When bouillon was added to sucrose the longevity and egg deposition reached a maximum. Sherman and Campbell (1928) when feeding two groups of rats on adequate diets which were nutritionally equal but differed in the proportion of the milk content found that the group receiving the larger portion of milk averaged a 10 per cent longer life span. Campbell (1928) has also shown that when rats were fed on a diet composed of 1 part milk and 2 parts whole ground wheat, and containing 15 per cent protein one-half of which was derived from the milk and the other half from the wheat strong healthy progeny resulted up to the 24th generation. A second group fed 1 part milk to 5 parts whole wheat and containing 13 per cent protein one-third of which was from milk and two-thirds

from wheat, had only reached the 17th generation. A third group fed milk 1 part and wheat 9 parts, containing 12 per cent protein, 20 per cent of which was furnished by milk and 80 per cent by wheat, bred so poorly that they were not continued beyond the second generation. The first group had the longest life span. Hitchcock (1926) experimenting on two groups of rats fed 1, a well-balanced diet containing 29 per cent protein, and 2, the same diet to which each rat received daily in addition 8 grams lean meat cooked rare. This diet contained 36 per cent protein. He found a greater mortality in the last group receiving the meat in addition. This is sufficient to show that sometimes slight changes in the ingredients of the diet may result in pronounced modifications of the physiological functions of the animal which may influence succeeding generations.

Life span. In order to determine what effect the different per cents of protein would have on the length of life all the original animals were fed on their respective diets until death occurred. The average weights and ages at death of both sexes in each group are given in table 1. This shows that in the males the greatest average death weight was found in group II, 222 grams. This was followed in a descending order by group V, 214; IV, 202; I, 192; and III, 191. The females showed a different order of death weights. From greatest to least they were III, 198; II, 185; IV, 167; I, 159; and V, 158 grams. These death weights show that the animals fed a diet containing approximately 14 per cent protein were able to maintain a heavier weight throughout life than any of the other groups.

The experiments of Burge (1923) may give an explanation for this difference. He concludes "a high protein diet produces an increase while low protein and starvation produces a decrease in the catalase content of the entire animal, in keeping with the fact that a high protein diet increases metabolism while low protein and starvation decrease it." We are now determining the B. M. R. of our different groups of rats and will give the results in a later paper. Table 1 also shows that the longest average life span in both sexes was in group II and that the shortest was in group V. The order in the males was II, III, I, IV and V and in the females it was II, III, IV, I and V. It is noted that the females had a longer average life span than the males in each group. The greatest difference of 91 days was in group IV and the least of 50 days in group III. The females of all the groups had an average life span 73 days longer than that of the males.

Table 1 does not give the range between the age at the earliest death and that of the oldest animal in each group. These data as well as the average life spans are shown in graphic form in figure 1. The heavy lines represent the average duration of life and the two light lines show the earliest and latest death of each sex in each group. This range in the males from greatest to least was group III, 842 days; V, 808; IV, 783; II, 770; and I, 745 days. In the females they were IV, 1054; V, 1047; I, 976; II, 707; and

III, 585 days. The oldest age attained by the males from greatest to least was II, 1172; V, 1152; III, 1128; I, 1086; and IV, 1057 days. For the females they were V, 1282; IV, 1276; I, 1250; II, 1108; and III, 1074 days. The average age of the five oldest males was 1119 days and of the five oldest females it was 1198 days, or 79 days longer than that of the males. Figure 1 also shows that the average life span of each sex of a group had practically the same relationship to the same sex of the other groups and that group II had the longest average life span and that group V had the shortest. This indicates that the optimum protein content for longevity was a little greater than 14 per cent.

Cause of death. In order to determine as nearly as possible the cause of death autopsies were performed and macroscopic examinations made in regard to the general external condition of the body and to such internal structures as lungs, heart, liver, alimentary tract, kidneys, and reproductive organs. These autopsy records were filed for further use. Since no

TABLE 1

Average weight and age at death of each sex in each group of the first matings

GROUP	MALES					FEMALES				
	Number	Weight		Age		Number	Weight		Age	
		Average	P.E.	Average	P.E.		Average	P.E.	Average	P.E.
I	25	192	±1.85	700	±3.69	24	159	±0.96	762	±7.18
II	23	222	±1.43	767	±5.90	21	185	±1.20	848	±6.98
III	22	191	±1.44	760	±7.21	21	198	±2.17	810	±6.22
IV	23	202	±1.72	675	±5.72	23	167	±2.23	766	±7.42
V	27	214	±1.65	650	±3.03	26	158	±1.18	730	±7.28

microscopic tests were made only lesions which were more or less conspicuous were noted.

Different investigators have reported various effects of high protein diets. Moore and Hitchcock (1930) in studying the effect of a luxus consumption of meat upon the kidney fed one group of control rats on a diet containing 29 per cent protein and another group a diet having a protein content of 36 per cent. They found that the high protein group was 20 per cent heavier, that the average daily urinary nitrogen was almost double, and that the weight of the kidneys was 14 per cent heavier than in the control group. A histological examination of the kidneys showed that the controls were normal while 74 per cent of the experimental group showed such pathological changes as "focal areas of fibrosis and cellular infiltration in the cortex, dilatation of the cortical tubules with hyaline casts, and slight to moderate thickening of Bowman's capsule." They do not consider these pathological changes analogous to chronic nephritis in man.

Newburgh and Clarkson (1923) found that diets containing meat produced renal injury in the rabbit. Since the rabbit is herbivorous in habits it cannot be used as a basis for comparison with an omnivorous animal. Later Newburgh and Curtis (1928) in studying the production of renal injury in the white rat by the protein in the diet concluded that both the level of the protein intake and the duration of the high protein feeding determined the degree of harm to the kidney, as measured by the occur-

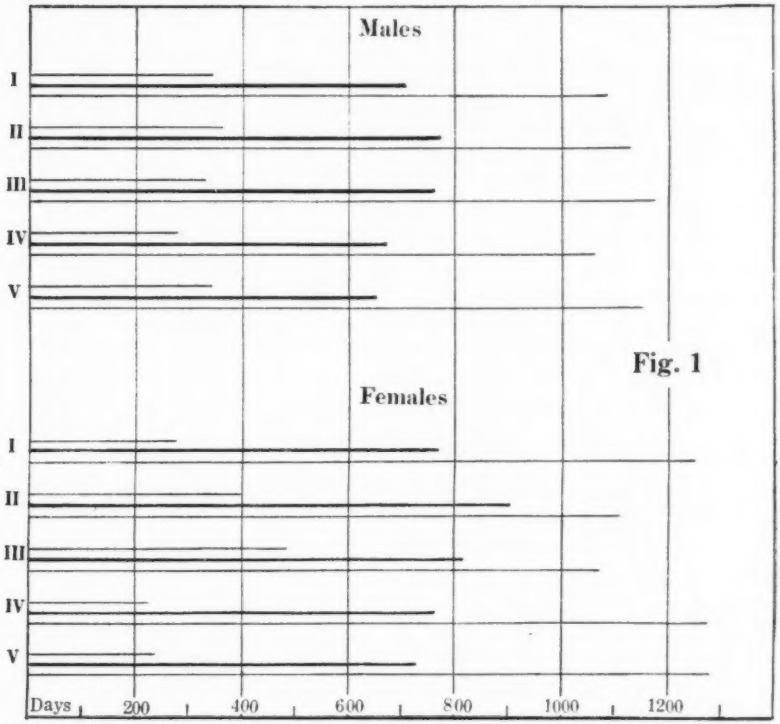

Fig. 1

Fig. 1. Showing the minimum and maximum life span in light lines and the average life span in heavy lines of each sex in the different groups, I, II, III, IV and V.

rence of casts. They believe that the differences in the amino-acid make up of the proteins may be the best explanation for the different amounts of renal injury which resulted.

In opposition to the above results we find a number of investigators who found that lesions of the kidneys did not follow a high protein intake. Addis and McKay (1926) using a diet containing as high as 69 per cent of protein found that though there was an increased excretion of nitrogen and

a hypertrophy of the kidneys in the group on the high protein diet they noted no microscopic difference in the structure of the kidneys of the control and experimental group of rats. These results were verified by the work of Osborne, Mendel, Park and Winternitz (1927) who studied the physiological effects of diets unusually rich in protein and inorganic salts. The investigations of Thomas (1927) and Heinbacker (1928) on the Eskimo showed that there was rarely any evidence of kidney disturbance and that rickets was not found in Greenland Eskimos. The results of Lieb

TABLE 2

Giving the per cent of various lesions found at autopsy in the males of the different groups of first matings

GROUP	NUMBER	LUNGS	ALIMENTARY TRACT	TUMORS		OLD AGE	MISCELLANEOUS
				Skin	Cancer-like		
I	22	100		4.54			
II	21	100		4.77			
III	17	82		5.87		11.75	
IV	14	100					
V	13	100					

TABLE 3

Giving the per cent of various lesions found at autopsy in the females of the different groups of first matings

GROUP	NUMBER	LUNGS	ALIMENTARY TRACT	TUMORS				OLD AGE	MISCELLANEOUS
				Mammary	Ovary	Uterus	Skin		
I	20	70	30.0	15.0	10.0		5		
II	19	58	10.5	10.5	21.0				5.26*
III	17	65	11.9	29.4				5.85	
IV	16	88			12.5				6.25†
V	21	67	14.3	4.8	14.3				

* Dead fetuses.

† Hemorrhage.

(1929) and of McClellan and DuBois (1930) on two arctic explorers substantiate the results on the Eskimo. No effect on the mental or physical ability was noted nor were they able to discover any specific physical changes in any of the systems of the body. The experiment, however, lasted but one year.

These references are sufficient to show that investigators do generally agree in that a high protein diet produces increased urinary nitrogen and hypertrophy of the kidney but disagree in regard to the harmful effects on

this organ. Little or no mention is made in regard to the effect on other organs or systems of the body. Since we made no histological study of any of the organs of our rats we are unable to state whether any microscopic lesions of the kidneys occurred. There was no macroscopic evidence of such injury present.

In tables 2 and 3 we have given the per cents of occurrence of abnormal conditions of various organs and systems in the males and females of each group respectively. The autopsies often showed that more than one abnormal condition was found in which case both were listed as contributing to the cause of death. By our method of autopsy it was impossible to determine accurately the true cause of death. The abnormal conditions found are, therefore, only suggestive contributory causes of death. There were very few animals in which no disturbance was noted. These we considered as having died of old age.

The most frequent disturbance was found in the lungs. They were often badly congested and suggested pneumonia. In some cases they appeared tubercular in character. By comparing tables 2 and 3 it is seen that lesions of the lungs were more prevalent in the males than in the females. The per cent of occurrence of lung trouble in each sex in the different groups does not suggest in this respect any effect of the different per cents of protein used. Lung trouble is a common disturbance especially in old rats and it is not surprising that we found it so frequently in our autopsies.

Another frequent disturbance was found in the alimentary tract—especially the large intestine and colon. This consisted of more or less constipation accompanied by dilatation of the intestinal lumen to often two or three times the normal. This dilatation was due to accumulation of gas formed probably by gas-forming bacteria. The wall of the tract was often so stretched as to be almost transparent. This disturbance was not found in the males to a noticeable degree. The females of group I had the largest per cent of lesions of the alimentary tract. This may have been due to the relatively greater amount of cellulose in the diet.

A third abnormal condition and often a large contributing factor to the cause of death was the presence of tumors. These consisted of varying sizes and were found in different locations. The range in size was from that of a small marble to as large as to constitute one-half the weight of the animal at death. They often became cancer-like in appearance. Tumors were developed only occasionally by the males and were found only in groups I, II, and III. They were of frequent occurrence in the females and present in all groups. Table 3 shows that the greatest numbers were found in the females of groups I, II, and III and the fewest in IV and V. Since the same relation existed in the males there is a suggestion that high protein diets are less favorable for tumor growth than low protein diets.

Table 3 shows that almost all the tumors in the females were associated with organs of reproduction. A slightly smaller per cent were found in the mammary glands than associated with the ovaries. The connection of these organs with tumors is suggestive of a correlation between them and tumor growth. In a former paper (Slonaker, 1930) we showed the close relationship between the ovaries and tumor growth which suggested a possible cause. Since tumors usually begin to form about the time reproduction functions cease and appear more prevalent in the female than in the male rat there is a strong suggestion that the cessation of normal ovarian function may play an important part in this growth.

SUMMARY

When five groups of rats, I, II, III, IV and V, were fed throughout life on diets containing 10, 14, 18, 22 and 26 per cent protein respectively the following results were obtained:

1. The greatest average maximal weight and the greatest average spontaneous activity were attained on the 14 per cent protein diet and the lowest on the 26 per cent.

2. The highest per cent of fertility in both sexes was found in group II and the lowest in V.

3. The length of the reproductive span from greatest to least was in the order of groups II, I, III, IV and V.

4. The average number of litters, the average size of the litters, and the average number of young per pair from greatest to least was in the order of groups II, I, III, IV and V.

5. The mortality of the young while nursing was least in group II and greatest in V.

6. Best growth of the young during lactation was attained on the higher per cent protein diets.

7. The loss of weight of the mothers during lactation was greatest in group I and became less as the per cent of protein in the diet increased. The high per cent of protein was best for both mother and the growth of the young during the nursing period.

8. The average life span from longest to shortest in both sexes was in the order of group II, III, I, IV and V.

9. The most frequent disturbance associated with death was lung trouble. This was more prevalent in males than in females. No correlation between this ailment and the per cent of protein in the diet could be made.

10. Lesion of the alimentary tract was more prevalent in the females and greatest in group I.

11. Tumors were more frequent in the females than in the males. Only the males of groups I, II and III had tumors. The females of groups I,

II and III had the greatest per cent of tumors. This suggests that high per cent protein diets are less conducive to tumor growth. Almost all the tumors in the females were associated with the mammary glands and ovaries.

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THE MAINTENANCE OF IRIS SPHINCTER TONE IN THE RAT

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Koppanyi and Sun (1926) observed mydriasis after instillation of strophanthin solution into the eye of the rat. The action of strophanthin, in this respect, was correlated by Barnard (1928) with the properties of the substance in lowering the surface tension of its aqueous solutions. Koppanyi (1927) found that irritants such as dilute acetic acid, caused pupillary constriction when instilled.

Barnard noticed that all the surface active substances used by him (saponin, digitalis, strophanthin, bile salts and resorcinol) were productive of corneal anesthesia, as well as of mydriasis. He also found that stimulation of the cornea by a weak tetanizing current or by blowing on the cornea, caused miosis, particularly if the rat made an attempt against resistance, to narrow its palpebral fissure. These findings were not previously reported, but from them it seemed possible that stimulation of the cornea, conjunctiva or periorbital tissue might in some way participate in the maintenance of oculomotor tone in the rat.

EXPERIMENTS. Adult white albino rats without pupillary inequality were used. The substances for instillation were placed into the conjunctival sac at intervals of three minutes until the desired effect was secured. The degree of mydriasis was approximated as slight, moderate and maximal. Corneal and conjunctival anesthesia was tested qualitatively by means of a fine wire, the absence of the winking reflex being used as a criterion.

RESULTS. All topical anesthetics tried, whether surface active or not, induced mydriasis (table 1) whereas 1 per cent apostesine (a local anesthetic not applicable topically) is ineffective in causing mydriasis when applied to the cornea of the rat.

Section of the ophthalmic branch of the trigeminal nerve, in three rats, resulted in complete anesthesia and moderate mydriasis in the eye on the operated side. The same results followed the ablation of the gasserian ganglion in two animals. Injection of 2 per cent butyn solution into the gasserian ganglion in 5 animals caused the immediate appearance of moderate mydriasis and complete anesthesia of the eye on the homolateral side, which disappeared simultaneously in about one hour.

The mydriasis produced by corneal anesthesia alone is less than that following anesthetization of cornea and conjunctiva. In one animal 0.02 per cent nupercain solution was instilled until the cornea was anesthesized but a winking reflex could still be elicited by touching the lid margin. The pupil then measured 2 mm. in white light and 3½ mm. in red light. (The normal rat's pupil is from ½ to 1 mm. in white light and 2½ to 3 mm. in red light.) The instillation of the anesthetic was continued in this animal, until exophthalmos existed and it made no attempt to close the eye, even after stimulation of the skin around the eyeball. The pupil then measured 3 mm. in white light and 4½ mm. in red light.

Section of the cervical sympathetic nerve in ten animals in no way interfered with the pupillary dilatation produced by the instillation of hexylresorcinol.

TABLE 1

SUBSTANCE	ANIMAL	NUMBER OF ANIMALS	METHOD OF ADMINISTRATION	DEGREE OF MYDRIASIS	DEGREE OF ANESTHESIA
Hexylresorcinol (1–1000)..	Rat	10	Topically	Moderate	Maximal
Hexylresorcinol (1–1000)..	Mouse	3	Topically	Moderate	Maximal
Cocain (5 mgm./kilo)....	Rat	1	Intraperitoneally	None	None
Cocain (10 mgm./kilo)....	Rat	1	Intraperitoneally	None	None
Cocain (25 mgm./kilo)....	Rat	1	Intraperitoneally	Slight	None
Cocain (100 mgm./kilo)...	Rat	1	Subcutaneously	Moderate	None
Apothesin (1 per cent)....	Rat	1	Topically	None	None
Butyn (2 per cent).....	Rat	6	Topically	Moderate	Maximal
Tutocain (5 per cent).....	Mouse	2	Topically	Moderate	Maximal
Pyridium (2 per cent)....	Guinea pig	1	Topically	Slight	Moderate

Discussion. These results indicate that pupillo-constrictor tone in the rat is maintained partially, at least, as a reflex involving afferent components of the ophthalmic nerve. That the anesthesia of the cornea does not reflexly inhibit the sympathetics, is indicated by the results on the rats in which the cervical sympathetic had been cut.[1]

Although the rat has a concensual light reflex, the divergent optical axes make binocular vision impossible. As Byrne has shown in cats, the accommodation reflex has its origin in the extrinsic musculature of the eye, for which the propioceptive fibres course through the ophthalmic branch of the trigeminus. Since the rat has a near point of vision of about eight centimeters (Lashley, 1931) accommodation for distance is probably absent. It is possible that the reflex pathways for maintenance of iris tone in the rat

[1] Koppanyi (1929) has shown that the innervation of the pupil in the rat is the same as that of man.

and those concerned with accommodation in the cat are phylenogenetically related.

The mydriasis induced by anesthesia of the rat's eyeball is not accompanied by loss of light reflex. In no instance is such mydriasis maximal unless the animal is placed in the dark. The pupillary tone is apparently a summation of impulses generated in the retina by light and in the sensory structures around the eyeball. The nature of these latter impulses is probably tonigenic. If we assume that the cornea of the rat like that of man is sensible only to pain, the constriction of pupil attendant on such stimulation as blowing upon it, is probably associated with the animal's attempt to close its eyes, and the frustration of this effort sufficiently stimulates the sensory receptors within the muscle to result in reflex miosis.

SUMMARY AND CONCLUSION

The iris control in the rat differs from that of the dog, rabbit and man in having a mechanism, the tone of which is controlled, in part, by sensory impulses arising in the cornea, conjunctiva and periorbital tissues. The effect of surface active substances in causing mydriasis after local application is due to their anesthetic properties.

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THE ABSORPTION AND EXCRETION OF WATER AND SALTS BY THE ELASMOBRANCH FISHES

I. Fresh Water Elasmobranchs

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It is well known that the blood, the tissues and the body-fluids of all elasmobranch fishes that have been examined contain urea in quantities ranging from 1800 to 2500 mgm. per cent.[1] No other vertebrate or invertebrate is known which normally contains such large amounts of urea, and the only instance of record where this substance accumulates in the body to this extent is the estivating lung-fish, in which excretion is completely suspended (Smith, 1930b).

Baglioni's (1906) view that this urea bears some special functional relation to the heart and other tissues appears to us to be effectively refuted by the criticisms of Bottazzi (1906) and the experiments of Fredericq (1922). Kurkenberg's (1888) belief that the tissues possess some special affinity for urea was adequately answered by von Schroeder, and the alternative theory subscribed to by numerous investigators, including von Schroeder (1890) and later MacCallum (1910), that the uremia results from the inability of the kidneys to excrete the urea, is for many reasons hardly more tenable.

Fredericq (1903) approached what we believe to be the crux of the problem when he emphasized osmotic pressure and pointed out that because the urea freely permeates the tissues it is osmotically indifferent toward them; but because it does not freely penetrate the gills and integument, it is osmotically active toward the *milieu exterieur*. In our earlier work with elasmobranchs we had come to the tentative view that the urea plays a direct and important rôle in the osmotic relations of these fish to their environment, but the proof of this theory had to wait upon more detailed information.

[1] Lack of space prohibits the inclusion here of a résumé of the extensive literature on the osmotic pressure and urea content of elasmobranch blood. It is hoped to publish this résumé at another time.

It may be noted, however, that the blood in these animals is hypertonic to the external medium (Duval, 1925a, b) and this circumstance places them in a superior position osmotically with respect to sea water, and reverses qualitatively the osmotic relations characterizing the teleost fish, the blood of which has an osmotic pressure lower than that of sea water. That the urea, which furnishes so large a part of this osmotic pressure, is directly and physiologically related to the absorption of water can hardly be doubted from the *prima facie* evidence. Nor can it be doubted that further knowledge of the elasmobranchs will illumine not only the unique biochemical problem of natural uremia which is peculiar to them, but also the broader problem of the evolutionary history of body-fluid regulation in the vertebrates generally.

The problem obviously presents many technical difficulties, and after we had made observations over a period of several years on marine elasmobranchs we felt that a final clarification could be effected only by examining these fish while living in a fresh-water habitat.

Through the favor of the John Simon Gugenheim Memorial Foundation, Mrs. Smith and myself were enabled to examine certain fresh-water elasmobranchs in Siam and Malaya; this work is reported in the present paper. Work upon marine elasmobranchs will be reported in a subsequent paper.

All previously recorded investigations upon elasmobranchs have been made upon animals which were living naturally in salt water ($\Delta = 1.50$ to 2.3°C.). The reason for this is, of course, that these fishes are predominantly marine today. There are good reasons for thinking that this marine habitat is secondary and that the elasmobranchs of the Silurian-Devonian periods were largely and primitively inhabitants of the continental fresh waters (cf. Marshall and Smith, 1930). A number of living elasmobranchs are known to migrate into fresh water (Weber, 1894; Englehardt, 1913; Beebe, 1925) or to live permanently there (Boulenger, 1922).

It is interesting to note that in the Munich collection Englehardt reports the following from fresh water: *Carcharias*, 4 species; *Sphyrna*, 1 species; *Pristis*, 4 species; *Rhinobatus*, 1 species; *Raja*, 2 species; *Taenuria*, 5 species; *Trygon*, 4 species; *Ellipesurus*, 1 species; total 22 species.

It is unlikely that this list exhausts the forms which breed or occasionally invade prefectly fresh water, and there are no reasons for supposing that any elasmobranch, under proper ecological conditions, might not perform this migration back to the ancestral home of the race.

Most of the work reported in this paper was done upon the saw-fish, *Pristis microdon* Latham (*P. perrotteti* in the older nomenclature).

This saw-fish is a frequent invader of fresh-water rivers in the tropics and was obtained by us in large numbers at Teluk Anson, Perak, F.M.S.

Teluk Anson Town, (village of Durian Sebatang) is about 40 miles from the mouth of the Perak River and at no time during our stay there did we detect any chloride in the water by silver precipitation. A careful salinity survey of the river was made, examining both bottom and surface samples at or near high tide. On the day of the survey the tidal range at Teluk Anson was 10 feet, though the spring tides may run as high as 13.5 feet. The survey was carried out by progressing up-river from the Straits of Malacca by motor boat, and timed to pass high tide a few miles above the river mouth. It was found that salt water shelves in on the bottom of the river while fresh water floats out on the surface of the sea; there was no detectable chloride (< 1.0 mM. per liter) on the bottom at Kuala Dedap, 28 miles below Teluk Anson, while the top layer showed 30.2 mM. of chloride at Bagan Datoh, which is just within the geographical limits of the mouth of the river (39 miles below Teluk Anson). The fish upon which we worked were caught within a range of three miles up and down the river from the village of Durian Sebatang, which was itself 28 to 30 miles from the *last trace* of salt water on the day of our survey. *Pristis* occurs in considerable numbers at Teluk Anson and for 50 miles up-river, frequenting the shallower water (6–8 feet), and there is every reason to believe that it is entirely habituated to this fresh-water habitat. Specimens of *Pristis* as large as 60 pounds have been caught at Teluk Anson, though the largest which was caught during our stay was 30 pounds. For experimental work we chose specimens ranging from 3 to 10 pounds because we were unable to keep larger ones in captivity.

The bamboo basket or "bubu" which is customarily used by the Malays to transport live fish was used by us to keep *Pristis* in captivity. For the most part we had no difficulty keeping the saw-fish in apparently good condition for several days. Since they were caught in nets in the shallow water where they fed, they were brought to us uninjured. No attempt was made to feed the fish in captivity, and, in view of the feeding habits of this animal, we doubt if such attempts would have been successful.

Specimens of *Dasyatis uarnak* (Forskal) and *Carcharhinus melanopterus* (Quoy and Gaimard) were caught at Teluk Anson. Both of these species customarily frequent the Perak River to points above Teluk Anson, but were very scarce while we were there. We were unable to obtain a sufficient number of them to do any experimental work upon them and confined ourselves to analyses of the body fluids, and to anatomical work upon the kidneys, which will be reported elsewhere. From local information we gather that there are four species of rays and three of sharks which at one time or another invade the Perak River well above salt water, some of them being of very large size, but we did not obtain others than those listed above. Undoubtedly there is both a ray and a shark which breeds in fresh water at Teluk Anson, because the eggs and young are correctly

described by the local fishermen. It is significant that no strictly marine teleosts occur in the river above Kota Stia, which is only a few miles from the sea; this demonstrates a greater tendency on the part of these elasmobranchs, as compared with the marine teleosts, to invade fresh water.

Dasyatis sephen (Forskal) was obtained at Lampam, Patalung Province, Siam, at the head of the Inland Sea. These rays have been observed to occur here in abundance in the past but we were able to procure only two live specimens. The water in which they were caught contained 14.2 mM. of chloride per liter, and was about 30 miles from a point where, at a narrow neck joining the upper and lower Seas, it becomes decidedly brackish.

TABLE 1

Composition of body fluids of fresh-water elasmobranchs

	Δ, °C.	MGM. PER CENT				mM. PER LITER			pH
		Non-protein N	Urea + NH_3-N	NH_3-N	T. creatine N	PO_4	SO_4	Cl	
Pristis microdon, ♂									
Blood	1.02	500	364	Tr.	1.6	3.1	None	170	7.5
Perivisceral f.	1.00	488	455	35.4	1.5	Tr.	None	204	<5.1
Pericardial f.			297	24.0	1.0			201	5.7
Bile	0.88		395	None				115	
Gastric f.	0.96		435	5.0		7.0		228	
Dasyatis uarnak, ♀									
Blood	1.02	406	292					212	
Perivisceral f.		314	252				None	278	
Pericardial f.		371	292					216	
Carcharhinus melanop., ♀									
Blood	0.90	423	288					158	
Hypolophus sephen, ♂									
Blood			228					146	7.5
Perivisceral f.									5.5

This ray, like *Pristis*, must be considered to be perfectly habituated to fresh water because it is known to breed in the upper reaches of the Inland Sea (personal communication from Prof. Hugh M. Smith).

Composition of body fluids. Analyses of body fluids from four species of fresh water elasmobranchs are given in table 1. The analytical methods used are described in an appendix to this paper. A number of analyses were made on *Pristis* which lack of space excludes; the data given in table 1 are typical and suffice for the present discussion.

The significant features of these analyses, in comparison with marine forms (cf. Smith, 1929a) are:

a. The osmotic pressure of the blood of fresh-water elasmobranchs, as judged by the lowering of the freezing point, is typically much lower (Δ = 1.0°C.) than in marine elasmobranchs (Δ = 1.85°–2.15°) but distinctly higher than in fresh-water teleosts (Δ = 0.7–0.8°C.).

b. The urea content of the blood is decidedly lower than in marine elasmobranchs but still distinctively high in comparison with other animals. We may take as typical values for the urea nitrogen, 300 mgm. per cent for fresh-water elasmobranchs, 1000 mgm. per cent for marine elasmobranchs and 10 to 30 mgm. per cent for other animals.

c. The chloride content of the blood of fresh-water elasmobranchs is decidedly lower than in marine elasmobranchs, typical values for the former being 170 mM. per liter and for the latter 230 mM. per liter.

d. Though having no bearing on the present discussion, it may be noted

TABLE 2

Composition of urine of Pristis in fresh-water

SEX	FASTED	Δ, °C.	MGM. PER CENT				mM. PER LITER						N:P
			Total N	Urea N	NH_3-N	T. creatine N	PO_4	Cl	SO_4	K	Ca	Mg	
	days												
♂	1	0.10	69.7	46.3	4.6	0.84	4.9	8.2					9.7
♂	2	0.10	81.0	57.4	4.6	0.65	3.0	5.1					4.8
♂	3		51.3	22.9	4.0	0.62	2.2	2.8					8.2
♀	0		56.9	31.6	4.2	8.70	12.6	1.0					35.2
♂	0		82.5	56.7	4.4	4.00	15.1	5.6					24.7
♂	0		82.0	44.1	5.0	7.80	8.9	None					18.1
♂	0		71.5	40.2	4.3	2.70	6.6	9.9	0.4	2.0	3.2	1.5	14.8
♂	0		62.0	44.5	3.0	2.55	6.1	13.1	0.3	4.0	1.1	0.9	12.8
♂	0		37.5	24.2	6.8	1.67	2.6	4.5	0.3	0.5	0.8	1.4	8.4

that the urea permeates all the body fluids, but is typically lower in the pericardial fluid than in the serum or perivisceral fluid (these usually have about the same urea content, contrary to the examples given in table 1); this lower urea content of the pericardial fluid recalls a similar distribution in marine elasmobranchs. The perivisceral and pericardial fluids are further distinguished, as in marine elasmobranchs, by the presence of ammonia and by an extraordinary acidity, indicating that they are not simple diffusates from the plasma.

The above facts show that the transition from salt to fresh water is accompanied by a fall in osmotic pressure (as determined by freezing point) of about 45 per cent, by a fall in blood urea of about 70 per cent and by a fall in blood chloride of 25 per cent. When viewed in relation to the blood of teleosts, the only significant difference in composition of the blood of

these fresh-water elasmobranchs is the persistence, though at a *much lower level*, of the uremia which characterizes the marine forms. It should be noted that the urea is reduced, relative to a marine level, to a much greater extent than is the total osmotic pressure or the chloride. This fact suggests that the uremia bears a special relation to a marine habitat, but that when the elasmobranch invades fresh water (even more or less permanently) the uremia is not entirely obliterated.

Composition of Pristis urine. Urine was collected from male sawfish by tying a glass retention catheter in the urinary papilla, the catheter carrying either a closed rubber bag or an open one attached to a long piece of rubber tubing. In the latter case the urine could be aspirated with a syringe from time to time without disturbing the fish. In the females a catheter was tied in the cloaca by a purse-string ligature, using every care to close the cloaca effectively around the catheter. No difference could be detected in the urine obtained by the two methods, and we feel sure that under the conditions of our experiments the female urine was not contaminated by intestinal contents or by urea from the cloacal walls.

Data on the composition of urine collected from animals on the day of capture and for three days thereafter are given in table 2. These data are typical of a great many which we collected. The significant features are:

a. No marked change in composition of the urine occurs in any one animal on successive days of fasting. We cannot assert that any of these specimens were in the active process of digestion, although food remnants were usually found in the stomach or intestines.

b. The urine is hypotonic to the blood, the freezing point in two specimens being —0.10°C. and the chloride content never rising to above 15 mM. per liter; the latter was usually below 10 mM. per liter in several dozen samples collected under a variety of conditions.

c. All constituents are very dilute, notably the urea which averages about 40 mgm. of urea N per cent and which is far below the blood level of 300 mgm. per cent.

d. SO_4 is present only in very small quantities. We think that sulphur must be excreted in some conjugated form, though we were never able to obtain significant SO_4 by hydrolysis with HCl.

e. PO_4 is present in much larger quantities than would be expected from the urea and ammonia content.

The first feature about the urine which we wish to discuss is the relatively low urea content. The average level of 40 mgm. per cent represents only 13 per cent of the average blood level. In one experiment we determined simultaneous values in blood and urine by drawing blood from an abdominal vein at the middle of a 2 hour period of urine collection. The blood urea N was 278 mgm. per cent and the urine urea N was 37 mgm. per cent.

The kidney of *Pristis* resembles that of other elasmobranchs in possessing

large (139 mica) and numerous (30,000 to 50,000 per kgm. of fish) glomeruli. We ascertained that this animal excretes both glucose and ferrocyanide, a capacity which Marshall (1930) has shown to be present in glomerular kidneys and absent in aglomerular kidneys. In the light of our knowledge of general kidney function and of the fact that the glomeruli permit the filtration of glucose and ferrocyanide in these fish, we are led to believe that urea is filtered into the glomerular capsule at about the blood level, and, since it is present in the urine at much lower concentrations, that it is subsequently reabsorbed by the tubules. (This conclusion would issue from the evidence already available on marine elasmobranchs, for they are known to have glomerular kidneys; Marshall (1930) has shown that *Raja erinacea*, *Squalus acanthias* and *Mustelus canis* excrete ferrocyanide, and Baglioni (1906); Buijtendijk (1909a, b); Denis (1912); Scott (1913) and Smith (1931) have invariably found that the urea content of the normal urine is less than that of the blood.) The belief that the urea is filtered and reabsorbed is reinforced by the experiments of Buijtendijk (1909b) in which medullary piqûre caused the urine urea to rise to about the level of the blood.

The second point of interest in the composition of the urine of *Pristis* in fresh water is the excessive quantity of PO_4, relative to the urea and NH_3—N, that is present in it. The excessive N:P ratio indicates that either urea or NH_3, or both, are being lost from the body by some route other than the kidneys.

We digress here to remark that before leaving New York, and with this end in view, we determined the N:P ratios (as mM. of P per 1000 mgm. of urea $+NH_3$—N) in a dog which had been fed canned salmon for two weeks; in a fasting dog, and in a fasting lungfish. The results are as follows:

Dog, fed salmon	2.77
Dog, fasted	2.65
Lungfish, long fasted	3.84

These figures fall within the range of those commonly observed in man. They are of value to us here because they indicate the approximate quantity of PO_4 which should accompany the urea and NH_3—N in the urine following the combustion of protein. We foresaw that part of the urea and NH_3 excreted by the elasmobranch might escape from the body by some route other than the kidneys, and we wanted some factor of reference in the urine to indicate if this were so. No other constituent of the urine is reliable for this purpose except SO_4, and our failure to obtain decomposition of the conjugated sulphates in the urine (if such they were) prevented the use of this substance. On the above data we may expect the N:P ratio to fall between 2.0 and 4.0 when all the urea and NH_3—N accompanies the PO_4 into the urine, and when no phosphorus-rich food is supplied.

The urines in table 2 were collected with every precaution to circumvent abnormal conditions. The sawfish from which they were removed were first-class specimens which had been caught on the previous night and carefully transferred from the nets to the "bubus" in which they were kept. The fish were catheterized as soon as they were removed from the water, only about two minutes being required for the operation. Sufficient urine was present in the 'bladder' for analysis. Yet the N : P ratio greatly exceeds the value of 4.0 (ranging, in fact, from 8 to 25) and this fact indicates that under normal conditions nitrogen is excreted from the body by some extrarenal route. This extrarenal excretion is discussed at length below.

Before leaving the discussion of the urine as such, it may be pointed out that the urine flow in *Pristis* when in fresh water is very large, even larger than we have observed in fresh-water teleosts (Smith, 1930a). In a large number of experiments we observed flows over a period of 3 to 4 hours varying from 150 to 460 cc. per kgm. per day, with an average value of 250 cc. (This is 50 to 100 times the urine flow in sea water as reported by various investigators.)

The extrarenal excretion of urea, ammonia and chloride. We were fortunately able to make very careful observations on the extrarenal excretion of *Pristis*. We constructed a galvanized iron tank about eighteen inches wide, twelve inches deep and six feet long in which individual sawfish, immersed in a known volume of reservoir water, could be kept for several hours. This water contained no significant quantities of urea, ammonia or chloride. The urine was collected by means of a retention catheter to which a soft rubber balloon and a long rubber tube were attached. The urine accumulated in the distensible bag and was aspirated from time to time without positive or negative pressure in the catheter itself. The water was aerated by dipping it out and pouring it back into the tank continually throughout the experiment. The shade temperature during most of the experiments described below was between 88 and 92°F., and the water temperature about 80°F. Data on a number of experiments of this nature are recorded in table 3. All these data are corrected to a *per kgm. per day* basis to facilitate comparison.

Experiments 11 and 12 record the results obtained on a normal male and female sawfish. The first horizontal column gives the excretion into the water, and the second gives the excretion into the urine.

It will be noted that most of the urea, ammonia and chloride are excreted into the water rather than into the urine. We were well aware of several dangers in this experiment and took care to circumvent these dangers in every possible way. In the first place, the peritoneal or abdominal pores of the elasmobranch fishes open directly from the peritoneal cavity to the exterior (cf. Smith, 1929a). The perivisceral fluid in this cavity is, of

course, rich in urea and chloride. We frequently observed that the perivisceral fluid was discharged from the peritoneal pores when sawfish were being handled, and we recognized the possibility that such discharge might occur during the course of our "fish-tank" experiment. We ascertained, however, that when a purse string ligature was placed around each peritoneal pore and pulled tight, no fluid could escape from the abdominal cavity. Consequently this precaution was taken in all experiments.

Secondly, we recognized that with such enormous urine flows there was a possibility that a kink in the catheter tubing might cause pressure within the catheter and result in leakage of urine. This was guarded against by the introduction of the soft rubber balloon between the catheter and the rubber tubing, and by testing the cather at the beginning and again at the end of the experiment by blowing air into it; any weakness of the ligatures could be detected easily in this manner. In addition, in one experiment, we injected phenol red intravenously; no trace of the dye could be found in the water after it was evaporated, and a simple calculation based on the relative amounts of phenol red, urea and chloride in the urine showed that the urea and chloride in the water could not have had a urinary origin.

Thirdly, *Pristis* contributes to the experiment by furnishing a convenient handle on his nose in the form of his saw, so that it is not necessary to hold the fish by the head or to compress the gills at any time. Since a slight mechanical injury to the gills may lead to hemorrhage (as sometimes happens when one is handling teleosts) we took every care to guard against such mechanical injury. It seemed to us, moreover, that some gill injury might result from asphyxia or from keeping the fish out of water too long, and consequently we endeavored to perform all our operations as quickly as possible. In most of the experiments listed here the total time that the sawfish was out of water did not exceed seven minutes.

In short, by the time we got to experiment 11 we had the technique of the experiment well in hand; yet, despite every precaution a large fraction of the excretory products continued to leave the body by some route other than the kidneys. Taking into account thirteen experiments, each of which lasted from 3 to 4 hours, we find that on the average:

a. Seventy-seven per cent of the total urea excreted by *Pristis* is excreted extrarenally. The minimum was 60.4 per cent and the maximum 84.3 per cent.

b. Eighty-nine per cent of the total ammonia is excreted extrarenally, with a minimum of 80.7 per cent and a maximum of 95 per cent.

c. Seventy-six per cent of the total chloride is excreted extrarenally, with a minimum of zero per cent (expt. 16) and a maximum of 100 per cent (expt. 21).

d. No extrarenal excretion of phosphate could be detected.

e. When the urinary PO_4 is compared to the *total* urea $+NH_3-N$, the

N:P ratio averages 2.75 mM. of P per 1 gram of N, a value consonant with our observations on the dog, the lungfish and the usual figures on man. This very significant fact indicates to us that in these "fish-task" experiments we are not getting an abnormal excretion of urea, but only the normal excretion which ordinarily is divided between the renal and extrarenal route. We are supported in this conclusion by the fact that the total

TABLE 3

Experiments on the extrarenal excretion of urea, salts, etc., in Pristis in fresh water

NUMBER		WEIGHT	HOURS	CC. PER KGM. PER DAY	MGM. PER KGM. PER DAY				mM. PER KGM. PER DAY		TOTAL UREA + NH_3-N, MGM. PER KGM. PER DAY	N:P RATIO
					Total N	Urea N	NH_3-N	T. creatine N	Cl	PO_4		
		kgm.										
11	Control ♂	2.27	3.5									
	Water					246	94	None	9.7	None	425	3.1
	Urine			190	136	77	8	5.2	1.9	1.3		
12	Control ♀	2.31	3.0									
	Water					264	74	None	3.6	None	497	4.0
	Urine			336	212	149	10	8.6	4.3	2.0		
19	Urea ♂	4.20	3.0									
	Water					188	129		4.8	None	407	2.7
	Urine			171	146	79	11	4.3	1.2	1.1		
14	NaCl ♂	1.97	3.7									
	Water					232	109		7.7	None	449	1.6
	Urine			156		100	8		3.2	0.7		
15	Na_2SO_4 ♀	1.28	3.5									
	Water					298	84		14.2	None	450	0.7
	Urine			161		56	13		1.9	0.3		
21	Water ♂	1.88	3.5									
	Water					250	111		11.7	None	533	4.3
	Urine			386		161	11	10.0	None	2.3		
22	$NaHCO_3$ ♂	2.24	3.0									
	Water					182	111		6.2	None	437	4.6
	Urine			458	296	119	25	8.6	0.3	2.0		
26	Glucose ♂	1.86	3.3									
	Water					174	88		None	None	384	3.0
	Urine			306		107	15		3.5	1.2		

urea $+NH_3$—N excretion remained quite constant in all experiments; it averaged 450 mgm. per kgm. per day—a figure that is not excessive for recently fed carnivorous fish kept at 80°F.

We tried in a number of experiments to modify this extrarenal excretion by injecting various substances into the animal. The results of several typical experiments are also given in table 3. We found that:

f. The proportion of urea which is excreted extrarenally was not sig-

nificantly modified by the intravenous injection of 0.75 mgm. of urea per kgm. in 30 per cent solution (expt. 19). Before injection the blood urea N was 300 mgm. per cent; 10 minutes after injection, 327 mgm. per cent, and at the conclusion of the experiment (3 hours) 327 mgm. per cent. Apparently the injected urea was taken up quickly into the tissues and a rise of 300 to 327 mgm. per cent in the blood did not significantly affect its excretion. Attempts to give larger doses of urea were frustrated by convulsions and death. The quantity injected in experiment 14 was about three times the quantity excreted during the course of the experiment.

g. The intravenous injection of 1.0 gram of Na_2SO_4 per kgm. resulted in the abundant excretion of SO_4 by the kidney, the urine containing 87 mM. per liter, or a total of 274 mgm., about one-third of the injected sulphate. There was no extrarenal excretion of sulphate.

h. The intraperitoneal injection of 40 cc. of distilled water, followed by 30 cc. similarly injected 2½ hours later, was accompanied during a period of 3½ hours after the second injection by marked extrarenal excretion of chloride and a chloride-free urine. The increase in chloride excretion is not significant since a lower value was obtained on repetition of the experiment, but the disappearance of chloride from the urine is possibly significant of more effective renal conservation of this substance.

The foregoing experiments are typical; they were all done in duplicate and some of them in triplicate. The animal showed an extraordinary and disappointing lack of responsiveness to injections of any of these substances. We recognized that smaller but significant changes in excretory activity might be observed in experiments in which the experimental period was preceded by a control period, but we were not successful, for reasons which need not be detailed, in carrying such experiments to a successful culmination.

In our previous observations on fresh-water teleosts we came to the conclusion that the extrarenal excretion of urea (by the gills) was a process of passive diffusion (Smith, 1929). This conclusion has been reinforced by our observations on the lungfish (Smith, 1930b). The observations which we have described above on *Pristis* likewise indicate that the extrarenal excretion of urea is a process of passive diffusion, for urea continues to leave the body at a fairly constant rate (200 to 250 mgm. of urea N per kgm. per day) under diverse conditions, although the renal excretion may vary three-fold.

The extrarenal excretion of ammonia and of chloride is more variable, however, and appears to be amenable to physiological regulation. This variability is brought out in several experiments which were primarily intended to elucidate other points. For example, in two experiments (nos. 17 and 20, not included in table 3) we transferred a sawfish from fresh water to sea water which had been obtained from the Straits of Malacca,

in order to observe the effects upon urea excretion. The sea water had a chloride content of 515 mM. per liter, and a pH of 7.6; phenol red was added to it in order to determine whether the fish swallowed any of the solution. It is important to note that extrarenal excretion of ammonia was markedly reduced, falling to 40 and 9.5 mgm. per kgm. per day respectively, which is well below the average level of 110 mgm. per kgm. per day. But the extrarenal excretion of urea was increased to 472 and 672 mgm. per kgm. per day. One fish survived the experiment and for 24 hours afterward. The other fish was sacrificed and the gastric and intestinal contents examined for phenol red with negative results. We can say that the fish did not drink any appreciable quantity of sea water in three hours, in spite of the fact that the immersion in this medium sufficed to completely arrest urine formation and therefore, by inference, water absorption.

It is very significant that urea continued to be excreted extrarenally under conditions which sufficed to completely arrest urine formation, and under just those conditions in which we would expect it to be conserved in order to bring the animal into that state of uremia which characterizes it in a marine habitat. (The conditions of the experiment were not such as to permit us to follow the effects of sea water upon the extrarenal chloride excretion.)

Recognizing the fact that the sea water was slightly alkaline (pH 7.6) and that this alkalinity may have been the immediate cause of reducing the ammonia excretion, we performed one experiment (no. 22) by adding sodium bicarbonate (10 grams in 35 liters) to fresh water. This had no effect upon the excretion of urea, ammonia or chloride. Since this experiment constitutes an excellent control it is included in table 3.

In another experiment (no. 23) we injected 6 cc. of a 1 per cent sodium bicarbonate solution intravenously and 4 cc. subcutaneously; the fish died at the end of the experiment, but in 2 hours had excreted extrarenally only 6 mgm. per kgm. per day of ammonia (in comparison with the average of 110 mgm.) although the extrarenal excretion of urea was unaffected. We were not able to repeat this experiment, but the result is quite emphatic and points to physiological control of the excretion of this substance. Further work is planned on this aspect of the problem.

In experiment 26 (table 3) a sawfish which had been fasted for 4 days received on the fifth day 3 grams and 4 grams of glucose in two intramuscular injections separated by six hours. A third injection of 5 grams was given on the morning of the sixth day just before the fish was catheterized and placed in the "fish-tank." We wished in this experiment to effect a reduction in urea excretion by sparing the combustion of protein; this result was accomplished to perhaps a slight extent, for the total urea and NH_3 excretion was the lowest ever observed. But the fact that the

extrarenal urea excretion (174 mgm. urea N) was still large supports our conclusion that this is a process of passive diffusion from the high blood level.

It is again significant, however, that in this experiment the extrarenal excretion of chloride stopped entirely. This result is difficult to interpret in relation to the glucose itself. Injections of glucose into fish which had been fasted for only one day did not affect the extrarenal excretion of chloride; and this makes us think that it was the fact that the fish in experiment 26 had been fasted six days, rather than the fact that it had been injected with glucose, that was responsible for the decreased chloride excretion. But the experiment is valuable in that it shows that the *extrarenal excretion of chloride and urea can be dissociated*, the chloride excretion disappearing completely while the urea continues to leave the body at a steady rate. We tried very hard to repeat the experiment on a long-fasted fish, but unfortunately our work was terminated by the onset of heavy rains which, by washing some poisonous substance into the river, made it impossible to keep the fish alive in captivity. We can only submit it, therefore, as an interesting but unconfirmed observation.

In regard to the chloride excretion, the total chloride in eight experiments averages about 20 mM. per gram of urea+NH_3—N. This value is about 3 times as large as was observed in a dog fed upon canned salmon (7.06) and 10 times as large as in a fasting dog (2.0); it is, however, impossible to evaluate its significance when we know nothing about the diet of *Pristis*, but we do not think that it necessarily indicates an abnormal loss of chloride from the body during our experiments.

The above experiments were disappointing in the failure to furnish more positive evidence of physiological control of these excretory processes, but they concordantly establish:

a. That under as nearly ideal conditions as it is possible to obtain with practice, very large fractions of the urea, ammonia and chloride excreted by *Pristis* in fresh water are excreted by some route other than the kidneys.

b. That when the total urea +NH_3—N is considered, the sum bears a proper or probable ratio to the phosphate excretion, and from this fact it may be concluded that urea and NH_3 constitute the principal end-products of protein combustion in this animal. When a small quantity of unidentified nitrogen in the urine is excluded, urea accounts for 65 to 85 per cent of the sum, with an average of 75 per cent. This fact also indicates that the extrarenal excretion of urea and NH_3 is a normal physiological process, a conclusion which is substantiated by the excessive N:P ratios observed in urines collected under as nearly ideal conditions as possible.

c. The extrarenal excretion of urea appears to be a passive diffusion from the blood, since no marked variations in the rate of its excretion could be effected under the conditions of our experiments; but the extrarenal

excretion of ammonia and of chloride appear to be under physiological regulation, since they can be dissociated from the urea excretion and completely, or nearly completely arrested.

The route of extrarenal excretion. That the integument, and more especially the branchial membranes, of the elasmobranch fishes are relatively impermeable to urea is self-evident from the fact that these tissues maintain as much as 2.5 per cent in the blood against the urea-free sea water which bathes them. This impermeability is all the more extraordinary when we consider that, with the exception of water, O_2 and CO_2, urea is one of the most diffusible substances in the body.

Duval and Portier (1923) have concluded from experiments with solutions of NaCl and urea that the elasmobranch gill is impermeable to urea. These experiments consisted of immersing the fish in various solutions and noting the change of osmotic pressure of the blood. Such experiments are without question adequate to demonstrate a low degree of permeability, but we doubt that they do or can demonstrate absolute impermeability. And we doubt that such absolute impermeability exists; entirely apart from the experiments recorded above, we consider it highly improbable that a thin epithelium of this nature should not permit diffusion of urea to occur to some small degree.

We have observed that most of the urea excreted by the teleost fish escapes from the body by an extrarenal route, and we have given reasons to think that this route is the gills (Smith, 1929b). In the teleost, the gills are apparently quite permeable to urea, and with the constant circulation of blood bearing 10 to 30 mgm. per cent through the branchial capillaries, it is not surprising that most of it should be excreted by this route into the respired water.

The anatomical situation is, of course, exactly the same in the elasmobranchs, and the diffusion gradient is greatly increased by a higher blood-level; our observations on *Pristis* lead us to conclude that they differ from the teleosts only in possessing branchial (and perhaps oral) membranes which are relatively less permeable to urea. We were unable to perform any "divided-box" experiments with *Pristis* such as we used to localize the urea excretion in teleosts, but it should be noted that the sawfishes and the sharks are invested by an integument of enamel denticles; these form a close-set covering over the whole body, including the head, the rostrum and even the surfaces of the fins. There is no abundant blood supply to any part of this integument, the chief vessels being those which supply the cellular pulp in the axis of the denticle spine and which enter through a central perforation in the under surface of the basal plate. So strong is this "shagreen" covering in *Pristis* that it can be cut by a sharp knife only with difficulty, and we had trouble in forcing strong needles through it when placing ligatures about the cloaca. A more perfect

natural armour could hardly be devised, and we doubt with all emphasis that the integument is a route of egress for the urea, ammonia and chloride which we have observed to be excreted extrarenally.

In contrast to the relatively poor blood supply to the integument, the gills, of course, receive the maximum circulation of the body. We cannot exclude from consideration the oral membranes, but even if a greater degree of permeability were assigned to these than to the gills, the greater blood flow through the latter would make it highly probable that they constitute the route by which the greater part of the extrarenally excreted urea escapes from the body.

The picture which we form of the fresh water elasmobranch, then, is an animal in which the gills, though *relatively* impermeable to urea, still permit a low grade diffusion to occur between the blood and the respired water. As an incidental diffusion process, it is not amenable to physiological regulation and not appreciably modified by moderate changes in the blood level of urea or by the osmotic or specific chemical equilibria of the organism.

This conclusion leads to a simple interpretation of the uremia which normally exists in these fish, for, as we have argued above, the composition of the urine leaves no room for doubt that the kidneys actively conserve urea (by tubular reabsorption from the glomerular filtrate, one presumes); and between renal conservation at one end, and low grade branchial diffusion at the other, urea tends to accumulate in the blood and to pervade the tissues and the body fluids in amounts exceeding those observed in animals in which the gills are more permeable (teleost fishes) or in which there is little or no renal conservation (Amphibia and Mammalia).

The anatomical arguments adduced above in favor of the gills as the probable route of the extrarenal excretion of urea apply with equal force to the extrarenal excretion of ammonia and chloride. But the available evidence leads us to think that we are not dealing with passive diffusion in the case of these substances, but with a physiologically controlled secretion.

Observations on marine elasmobranchs will be reported in a subsequent paper.

Methods. Freezing points were determined on 5 to 8 cc. samples by a Beckmann thermometer. No correction was applied for undercooling.

Non-protein nitrogen was determined by digestion by Koch and McMeekin's method (1924) and direct Nesslerization; urea by urease decomposition, and ammonia by Nesslerization, both with and without absorption on permutit; total creatine by Folin's method (1922); PO_4 by Briggs' modification of the Bell-Doisey method (1924); SO_4 by benzidine precipitation and titration (Fiske, 1921); Cl by Van Slyke's method (1923); pH colorimetrically; Ca, Mg and K were determined on samples returned

to New York, Ca and Mg by Blanchard's method (Smith, 1930a) and K by precipitation as the cobaltinitrite (Kramer and Tisdall, 1921) and diazotization (Briggs, 1923). The urine was analyzed directly.

Except for Cl and freezing point, a trichloroacetic acid filtrate was prepared for the analyses of the blood and body fluids by the addition of 3 volumes of water and 1 volume of 20 per cent trichloroacetic acid to 1 volume of whole blood.

SUMMARY

Analyses are reported on the composition of the body-fluids of the fresh-water elasmobranchs, *Pristis microdon* Latham, *Dasyatis uarnak* (Forskal) *Carcharhinus melanopterus* (Quoy and Gaimard) and *Dasyatis sephen* (Forskal).

The blood has typically a freezing point of —1.0°C., a urea-N content of 200–300 mgm. per cent and a chloride content of 170 mM. per liter.

The urine of fresh-water *Pristis* is very dilute and resembles the urine of fresh-water teleosts. The urea content of the urine is invariably less than that of the blood and reasons are given for believing that the kidneys of the elasmobranch fishes actively conserve urea by reabsorbing it from the glomerular filtrate. The urine flow averages 250 cc. per kgm. per day.

Considerably more than half the urea, ammonia and chloride excreted by *Pristis* escapes from the body by an extrarenal route. The evidence indicates that the extrarenal excretion of urea is a passive diffusion across the gills, while the extrarenal excretion of ammonia and chloride is a physiologically controlled process (probably also effected by the gills).

The uremia which characterizes the elasmobranch fishes in general is explained by renal conservation of urea on the one hand, and the low degree of permeability of the gills, oral membranes and integument to this substance on the other.

Urea is apparently a normal end-product of the combustion of protein nitrogen in the elasmobranch fishes as in the lungfish, the teleosts, the Amphibia and the mammals.

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THE ABSORPTION AND EXCRETION OF WATER AND SALTS BY THE ELASMOBRANCH FISHES

II. Marine Elasmobranchs

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An examination of fresh-water elasmobranchs has led us to the conclusion that, except for a low-level uremia, they are physiologically similar to fresh-water teleosts in regard to the regulation of the composition of the body fluids (Smith, 1931).

Before considering the relation of the marine elasmobranch to the fresh water forms, it will be advantageous to restate briefly the general osmotic problem; and the solution to this problem as effected by the teleost fishes.

The organism living in fresh water is osmotically superior to the external medium (i.e., its body fluids have the higher osmotic pressure) and therefore it can and will invariably tend to absorb water (and to lose salt) spontaneously in response to the existing osmotic gradient. The organism living in salt water is osmotically inferior to its environment and tends, therefore, to lose water (and to absorb salt) in response to the existing osmotic gradient. In order to compensate for incidental or requisite changes in water and salt content of the blood and tissues, the fresh-water organism is required to excrete a fluid which is *hypotonic* to the blood; while the salt-water organism is required to excrete a fluid which is *hypertonic* to the blood. From an energetic point of view there is little difference between these two processes, for both require that the organism do osmotic work by separating from the blood a salt solution of dissimilar osmotic pressure.[1]

[1] The gastro-intestinal tract may be excluded in this consideration, for it appears that in all vertebrates (including mammals) fluids introduced here tend only to come to osmotic equilibrium with the blood; that is, the gastro-intestinal tract has never, in the vertebrates, acquired the capacity to do osmotic work. (We use the term osmotic work here to mean the separation of two fluids of dissimilar total osmotic pressure; it refers, therefore, to the distribution of water rather than to the distribution of any particular solute.) An apparent exception to this generalization occurs in the cloaca of birds and reptiles where water is said to be reabsorbed against osmotic pressure, but if true, this seems to be a specialization peculiar to this extra-intestinal organ in these animals.

But, from a physiological point of view, the capacity to excrete a *hypotonic* urine appears to have been evolved separately and independently of the capacity to excrete a *hypertonic* urine. The capacity to excrete a hypotonic urine is typical of all vertebrates, including the elasmobranchs and mammals (cf. Smith, 1931; 1930; Brunacci, 1917; Bottazzi, 1906; Burian, 1910; d'Errico, 1907). But the capacity to excrete a hypertonic urine is absent from the teleosts (Rodier, 1899; Dekhuyzen, 1905; Bottazzi, 1906; Smith, 1930), amphibia (Brunacci, 1914), reptilia (Burian, 1910 and unpublished observations of our own) and only slightly developed, if present at all, in the birds (d'Errico, 1907); whereas it is well known that the mammals can excrete a urine that has several times the osmotic concentration of the blood. Thus the capacity to excrete a hypotonic urine appears to have been evolved very early in the vertebrate history (before the elasmobranchs) and suffices to maintain the osmotic pressure of the blood in organisms which live in (or freely ingest) fresh water; while the capacity to excrete a hypertonic urine appears to have been evolved in the mammals, or perhaps in the reptiles.

In the absence of hypertonic renal excretion the teleost, when it lives in salt water, is required to perform its osmotic work elsewhere. We have adduced evidence that this osmotic regulation is affected by the extrarenal excretion of NaCl and KCl. By this operation it is enabled to derive an isotonic or hypotonic urine, in compliance with the osmotic limitations of its kidney, by drinking the relatively concentrated salt solution in which it lives (Smith, 1930).

When the elasmobranch moves from fresh into salt water, its osmotic relations are also reversed, for it must now maintain its intrinsic water content and its specific salt content against the constant tendency of water to escape from the body and for salts to enter. We may consider that these two processes proceed, in principle, independently of each other, and that the organism can control them by independent means. It appears that such is actually the case in the elasmobranchs.

The osmotic pressure of the blood and urine in relation to the external environment. Duval (1925) has observed that the osmotic pressure of elasmobranch blood, as judged by the freezing point, is appreciably greater than that of the sea water to which they are acclimatized. The difference is slight, but very significant.[2]

The osmotic pressure of elasmobranch urine has been determined by several investigators (Bottazzi, 1906; Burian, 1908; Buijtendijk, 1909) and in general it has been found to be lower than that of the blood.

[2] It is recognized that the determination of osmotic pressure by the freezing point method is open to a serious criticism; due to differences in the temperature coefficients of the several osmotically active constituents it is not impossible for two fluids which might be isotonic at −1.0°C. to have different osmotic pressures at 15°C.

In none of these investigations has the osmotic pressure of the sea water, the blood and the urine of thoroughly acclimatized animals been determined simultaneously, and consequently we include in table 1 a series of such observations. The first eight fish were obtained at the Mount Desert Island Biological Laboratory and the next five through the courtesy of Mr. C. M. Breder of the New York Aquarium; a few data on *Pristis* in fresh water are included for comparison. In regard to the first group it may be noted that the freezing point of the water of Frenchman's Bay (Salisbury Cove, Me.) is very constant, showing in this series a variation of 0.014°C. These animals were kept in captivity (fasting) for four days to two weeks in order to assure acclimatization to the local water; they were

TABLE 1

Osmotic pressure of serum, water and urine in elasmobranchs in relation to habitat

NUMBER		Δ °C., SERUM	Δ °C., WATER	Δ °C., URINE	DIFFERENCE SERUM-WATER	DIFFERENCE SERUM-URINE
25	Raja stabuliforis	1.983	1.860	1.685	+0.123	+0.298
14	Raja stabuliforis	1.929	1.850	1.915	+0.079	+0.014
19	Raja diaphanes	1.887	1.864	1.827	+0.023	+0.060
20	Raja diaphanes	1.874	1.864	1.860	+0.010	+0.014
21	Raja erinacea	1.922	1.850	1.920	+0.072	+0.002
22	Raja erinacea	1.922	1.850	1.892	+0.072	+0.032
24a	Squalus acanthias	1.952	1.850	1.865	+0.102	+0.087
24b	Squalus acanthias			1.730		+0.222
5	Raja sp.	1.617	1.484	1.065	+0.133	+0.712
6	Raja sp.	1.607	1.484	0.895	+0.123	+0.712
4	Raja sp.	1.227	1.091	0.788	+0.136	+0.439
11	Raja sp.	1.366	1.091	0.536	+0.275	+0.830
12	Raja sp.	1.328	1.091	0.473	+0.237	+0.855
	Pristis microdon	1.02	0.0	0.10	+1.0	+0.92
	Marine teleost, typical	0.80	1.85	0.7	−1.0	+0.1
	Fresh water teleost, typical	0.70	0.0	0.1	+0.7	+0.6

bled as soon as they were removed from the water to prevent changes in osmotic pressure in the blood incidental to asphyxia. Of the animals in the second group, nos. 5 and 6 were collected and bled off Sandy Hook, and a sample of sea water was taken simultaneously. The water at this point is slightly brackish, due to dilution by the Hudson River. Nos. 4, 11 and 12 had been in captivity for some time in the New York Aquarium, and had been fasted for two weeks prior to bleeding. They had been kept in circulating water obtained from New York Harbor, which shows slight osmotic fluctuations with the tides and the wind, so that it cannot be said that they were perfectly acclimatized to the osmotic pressure shown. The

typical values for the teleost are arbitrarily chosen to show the well known relationships that characterize this group.

It will be noted that:

a. The osmotic pressure of the blood is always higher than the osmotic pressure of the external medium; a curve generated by the former as ordinate and the latter as abscissa begins with the blood at an intersect or "threshold" of $\Delta = 1.0^\circ$ in fresh water and approaches the bisectrix asymptotically. The nature of this curve is such that the blood is always hypertonic to the external medium, although in sea water and more concentrated solutions the difference becomes very slight.

b. The urine, on the other hand, is invariably isotonic or hypotonic to the blood, and frequently hypotonic to the sea water itself.

From these facts it appears that the marine elasmobranch is always in a superior position osmotically to its environment; in this respect, it is like a fish in fresh water. By virtue of this fact it can and will tend to abstract water from that environment in consequence of the existing osmotic gradient. It must therefore excrete a urine which is isotonic or hypotonic to its blood in order to dispose of the water which will tend to enter its body. (In point of fact, the potential hypotonicity of the urine is to some extent obscured by the addition of metabolites, and possibly by the ingestion of sea water itself. But even if sea water is swallowed, the osmotic pressure of the urine need not rise above that of the sea water because the abstraction of water from sea water by the organism's hypertonic blood is always operating to dilute the urine and to permit the excretion of the added substances.) Since the urine is actually observed to be hypotonic to the blood, we are permitted to infer that direct water absorption is actually occurring in accordance with these osmotic gradients. The marine elasmobranch, then, by keeping its blood hypertonic to the external medium in *sea water* as well as *fresh water*, is continuing to carry on its osmotic regulation by the fresh-water mode of direct water absorption and hypotonic urine excretion.

This "functional" hypertonicity of the blood of the elasmobranch results in large part from the accumulated urea; it can hardly be doubted that the urea is physiologically retained for this purpose. This fact is brought out more forcibly by the data of table 2 showing the osmotic pressure, the chloride content and the urea content of serum in relation to the osmotic pressure of the external medium.

Between fresh water and sea water ($\Delta = 1.85^\circ$C.) the osmotic pressure of the serum increases, in round figures, about 100 per cent; the chloride increases 60 per cent and the urea better than 300 per cent. The last figure probably does not represent the maximum variation because we have observed values for the urea N as high as 1300 mgm. per cent (Smith,

1929).[3] In looking for those features which tend to be maintained in a steady state, we may exclude both the urea content of the blood and the osmotic pressure, for both are permitted to vary (between a fresh-water and a salt-water habitat) over a wide range. The increase in urea is largely responsible for the increased osmotic pressure of the blood; and, we suppose, occurs for precisely that purpose—namely, to permit the organism to absorb water from sea water by a natural osmotic gradient. In this view we can say that the elasmobranch is *indifferent* to the urea content of the blood except as it subserves an osmotic rôle. But we may

TABLE 2

Composition of elasmobranch serum in relation to habitat

NUMBER		Δ °C., WATER	Δ °C., SERUM	Cl	UREA N
				mM. per liter	*mgm. per cent*
13	Raja diaphenes	1.850	1.928	254	937
15	Raja stabuliforis	1.850	1.930	273	937
16	Raja diaphenes	1.850	1.924	272	1,000
18	Raja stabuliforis	1.850	1.933	257	1,091
5	Raja sp.	1.484	1.617	224	865
6	Raja sp.	1.484	1.607	214	850
8	Raja sp.	1.484	1.710	214	845
3	Squalus acanthias	1.330	1.622	234	695
2	Dasyatis centrura	1.330	2.010	265	560
4	Raja sp.	1.091	1.227	183	708
11	Raja sp.	1.091	1.366	162	583
12	Raja sp.	1.091	1.328	173	529
	Pristis microdon	0.0	1.02	170	364
	Pristis microdon	0.0	0.93	169	261
	Dasyatis uarnak	0.0	1.02	212	292
	Dasyatis uarnak	0.0	0.92	177	286
	Carcharhinus melanopterus	0.0	0.90	158	288
	Carcharhinus melanopterus	0.0	1.02	169	251
	Hypolophus sephen	0.0		146	228
	Hypolophus sephen	0.0			189

extend the argument by saying that the elasmobranch is equally *indifferent* to the *osmotic pressure* of its blood except as that osmotic pressure also subserves the rôle of water absorption. So, in inquiring what feature is regulated to a "steady state," our attention shifts from the urea and the osmotic pressure and becomes focussed upon the essential feature—the water content of the body. There are many reasons for supposing that

[3] There are many data in the literature showing values much higher than this, but the urea was measured by older methods and is probably too high. It is to be expected that the Mediterranean elasmobranchs will show higher values than those given in table 2, however, because of the greater salinity of the water.

in the general problem of the regulation of the composition of the body, the central one is the maintenance of a definite water content. That this is true of the elasmobranch fishes is indicated, indirectly but forcibly, by the above facts.[4] To this, and as a secondary feature, we must add the specific salt content for, certainly in the teleosts and to a great extent in the elasmobranchs, this is kept within relatively narrow limits despite changes in the salt content of the environment.

These observations lead us, then, to this interpretation: that in relation to environmental variations the elasmobranch tends, first, to maintain a constant water content in its body and, second, a constant salt content in this water (or in the body fluids); the urea content and the osmotic pressure (as specific features) are only means toward the absorption and excretion of water—i.e., urea is retained, and the osmotic pressure of the blood is raised, in order that water may be absorbed and that the kidneys may excrete an isotonic or hypotonic urine, in compliance with their osmotic limitations.

This point of interpretation is an important one, for if osmotic pressure *per se* is a feature of physiological indifference among the elasmobranchs, may we not question its significance among fishes generally and even among all vertebrates? Should we not discard it from the realm of significantly "steady states" which Bernard envisaged in the *milieu intérieur*, and recognize it as mere concomitant of what are really the fundamental steady states—namely, a, water content (possibly with reference to the tissue proteins) and b, specific salt content?

Composition of elasmobranch gastro-intestinal fluids. The foregoing observations make it easier to approach the problem of the absorption and excretion of salts. Two questions arise immediately: Does the marine elasmobranch drink sea water constantly, as does the marine teleost, and are salts excreted extrarenally as in the fresh water elasmobranch and the teleost?

The first question cannot be answered by simple analyses of the gastro-intestinal contents or urine because a slight (and what we may call incidental) ingestion of sea water will inevitably occur in any animal living and feeding in this medium. We have, therefore, performed a number of experiments which were intended to answer this question directly. These experiments were carried out with *R. diaphenes*, *R. erinacea* and *A. vulgaris* at Salisbury Cove. The fish were placed in tubs of sea water aerated by compressed air, phenol red was added to the water, and at the end of 18 to

[4] This statement is a flat contradiction of the conclusions of Margaria (1930), who finds that marine elasmobranchs, when abruptly transferred to diluted sea water, absorb water (gain in weight) and retain this water over a period of a few hours. We view these results as due to the inability of the organism to effect the necessary readjustments in a short space of time.

24 hours the animals were sacrificed and the gastric and intestinal contents were examined for the dye. In a few instances traces of the dye were found in the gastric contents, but only rarely in the intestinal contents and never in the urine. This result is in marked contrast to the marine teleost, in which the dye is found abundantly in the stomach and in the intestine after an exposure of this length of time, and in which the dye is concentrated in the intestine several fold in consequence of the absorption of a large part of the ingested water. We conclude from these experiments that the marine elasmobranch does not habitually "drink" sea water, but only swallows it occasionally, and more or less by accident.

In the light of these results it appears that the marine elasmobranch absorbs its water (apart from the incidental or accidental ingestion of small quantities) through some exposed membrane; it is not unlikely that this route of absorption is the mucous membranes of the mouth and upper esophagus. In this respect the elasmobranch, whether in fresh water or salt water, again resembles the fresh water teleost.

We are now in a position to consider the fate of such salts as may gain access to the animal *via* the gastro-intestinal tract.

Data on the composition of the fluid contents of the stomach, the intestine and of the bile removed from marine elasmobranchs are given in table 3.

It will be noted that:

a. Urea is present in the gastric contents and generally increases in concentration in the intestinal contents. This fact is consonant with the belief that the urea tends to pervade all portions of the animal by simple diffusion; it rarely appears in any of these fluids in concentrations greater than in the serum (cf. last column, table 3). Because it diffuses so readily, it exerts but little osmotic pressure between these fluids and the serum and therefore the total osmotic pressure (as measured by the freezing point) is not significant of the true osmotic equilibrium between them and the blood; this could only be evaluated relative to the non-diffusible constituents, such as salts, etc., and we have at present no method of doing this.

b. In practically all instances that we have examined (we have many data not recorded in table 3), Mg and SO_4 tend to become concentrated as the fluid passes from the stomach to the intestine, showing that the absorption of water, to the partial exclusion of these substances, is going on.

c. Since K, Ca and Cl do not become concentrated in the intestine to the same extent as Mg and SO_4, we may conclude that these substances (and Na, since most of the Cl is paired with it) are absorbed with the water.

Thus the evidence points to the same gastro-intestinal picture as has been described in the teleost (Smith, 1930) and as is known to occur in the mammals, namely, Na, K, Cl, Ca (and probably PO_4) and water are absorbed to the partial exclusion of Mg and SO_4. In addition, urea tends

TABLE 3

Composition of elasmobranch gastro-intestinal fluids

NUMBER		mM. PER LITER								MGM. PER CENT	
		Δ° C., water	Δ° C., blood	Δ° C., fluid	K	Ca	Mg	Cl	SO_4	PO_4	Urea N, fluid	Urea N, serum
3	S. acanthias, gastric	1.33		2.037	20	35	8	407	13		149	
	intest.		1.622	2.097	16	26	21	183	26		615	695
15	R. stabuliforis, gastric	1.85		1.880	23	14	16	379	18	None	798	
	intest.		1.930	2.092	5	17	63	309	63	None	1,125	937
17	R. stabuliforis, gastric	1.85		2.150	23	15	12	357	14	None	882	
	intest.		1.935	2.258	6	40	119	198	149	None	980	1,056
27	R. stabuliforis, gastric	1.85			19		20	317	7	None	1,023	
	intest.				Trace		196	311	151	None	978	1,091
15	R. stabuliforis, bile	1.85	1.930	1.947	3	16	None	210	None	None	952	937
16	R. stabuliforis, bile		1.924	1.847	3	16	None	157	None	None	1,333	1,000
17	R. stabuliforis, bile		1.935	1.967	4	20	None	240	None	None	962	1,056

to diffuse into the non-absorbed residue with osmotic indifference and thereby to raise the total osmotic pressure, which sometimes in the gastric contents and more frequently in the intestinal contents may exceed the total osmotic pressure of the blood and the ingested sea water (by a difference of 5 to 17 per cent). In view of the obvious circumstances that we begin with a salt solution (sea water) which contains salts in higher concentration than the blood, and that perfect equilibrium with respect to these salts is rarely obtained in the intestine, this slight hypertonicity cannot be interpreted as indicating that the intestine of the elasmobranch can absorb water against osmotic pressure; but only that urea has been added to a sea water residue the salts of which are still slightly hypertonic to the blood.

The composition of urine. The urines listed in table 4 were collected by aspiration from the bladder, with the exception of *Acanthias* in which the urine was collected by retention catheter.

a. Urea invariably occurs in the urine in lower concentration than in the blood, as has been noted by previous observers, and by ourselves in the fresh-water *Pristis*. The urine urea may sometimes be as low as 7 per cent (no. 30) of the blood urea. As argued in a previous paper (Smith, 1931), this result must in all probability be interpreted as due to tubular reabsorption of this substance from the glomerular filtrate.

b. The N:P ratio invariably exceeds the maximum of 4.0; but since some urea is lost from the body of marine elasmobranchs in the non-absorbable intestinal residue, it is doubtful if this fact can be interpreted to show branchial excretion of this substance. But when we consider that the extrarenal excretion of urea in fresh-water elasmobranchs is probably a diffusion process, and that the blood level of urea is increased 3- to 4-fold in sea water, there can be hardly any doubt that such extrarenal excretion occurs here as well. It could only be reduced, and not prevented, if the organism rendered its gills 3 to 4 times more impermeable to urea in sea water than in fresh water, and this seems very unlikely.

c. When in undiluted sea water ($\Delta = 1.85°$C.) Mg may appear in the urine in high concentrations; this we attribute to a slight absorption of this salt from the highly concentrated residue left in the intestine, as we have observed in the teleost. As the osmotic pressure of the external medium is decreased, the urine becomes more dilute, as would be expected from the relatively greater absorption and excretion of water. But the fact that in most instances the ratio of Mg to Cl in the urine of marine elasmobranchs exceeds that of the ingested sea water shows that chloride (and by inference Na and K) are being excreted extrarenally, as has been shown to occur in fresh-water elasmobranchs and teleosts. This ratio would necessarily vary from time to time, depending upon the degree of concentration of the sea-water residue in the intestine. The absolute concentrations of Mg in

TABLE 4

Composition of elasmobranch urine

NUMBER		Δ° C., WATER	Δ° C., URINE	mM. PER LITER						MGM. PER CENT			UREA N, SERUM	N:P*
				K	Ca	Mg	Cl	SO_4	PO_4	Total N	Urea + NH_2-N	T. creatine N		
14	R. stabuliforis, ♀	1.850	1.915	51	25	223	406	248	50	145	74	83	937	68
24a	S. acanthias, ♂	1.850	1.952	83	7	24	157	46	79	391	344			23
24b	S. acanthias, ♂	1.850	1.730	Trace	18	74	360	94	55	123	100			55
24c	S. acanthias, ♂	1.850	1.865	Trace	17	74	306	101	60	92	71			84
25	R. stabuliforis, ♀		1.685	91	13	104	64	44	79	545	500		695	16
30	R. stabuliforis, ♀			2	116	260	335	341	71	145	72		1,002	10
	D. imbricatus, ♀	(1.9)		32	None	33	184	56	42		755	5		6
4	R. sp.	1.091	0.788	22	6	25	25	45	66	521	167	104	708	40
6	R. sp.	1.484	0.895	23	5	8	81	18	45	410	365	29	850	12
11	R. sp.	1.091	0.536	5	7	2	20	19	4	460	420	33	583	10
	Sea water	1.850		9	9	50	483	30	None					

* 1 gram of urea + NH_2 − N = x mM. of phosphate. For significance of this ratio see Smith, 1931.

the urine will also vary considerably, since the animal derives its water primarily by extra-intestinal absorption, and only occasionally swallows sea water.

With regard to the urine flow, Baglioni (1906) observed 4.15 cc. per kgm. per day in *Scyllium catulus* and 5.25 cc. in *Scyllium stellare;* Burian (1908) observed 2 to 4 cc. in *Scyllium catulus;* Denis observed 18 cc. in *Mustelus canis* and Scott (1913) reports 21.6 cc. per animal per day in the same species; Hoskins and Hoskins (1918) observed 18 cc. per kgm. per day in *Acanthias vulgaris;* White (1931) 1.4 cc. in *Squalus scukleyi* and Marshall (personal communication) observed from 4.7 to 12.2 cc. in *Acanthias vulgaris* after 4 to 5 days of fasting. Since the urine flow will tend to be increased if the animal swallows any sea water, which may well happen when they are being pursued or returned to sea water incidental to catheterization, the upper figures may be too high. It would seem probable, then, that the normal flow is from 1.0 to 5.0 cc. per kgm. per day, or about one or two per cent of the flow which we have observed in *Pristis* in fresh water. This reduction in urine flow, we may suppose, results primarily from reducing the osmotic gradient between the blood and the external medium from 1.0°:0.0° in fresh water to 1.95°:1.85° in sea water. It may be secondarily modified, however, by the difference in the quantity of water which is excreted extrarenally in the two environments, for the extrarenal excretion of salt must require some water, and in sea water it is reasonable to suppose that more salt enters the body incidental to eating, etc., than in fresh water.

Discussion. We have adduced evidence that the marine elasmobranch by conserving urea through renal reabsorption, raises its blood to an osmotic pressure above that of the sea water in which it lives; it is thus enabled to absorb water from sea water to the exclusion of salts, and it then has this water available for the excretion of a urine isotonic or hypotonic to its blood, in compliance with the osmotic limitations of its kidneys. Thus by uremia it restores itself to those osmotic relations between the *milieu intérieur* and the *milieu extérieur* which characterize the fresh-water elasmobranch and the fresh-water teleost.

We are now in a position to compare the osmotic relations of the elasmobranchs to those of teleosts. We begin with the inference that in the biochemical economy of these fishes osmotic pressure is not important *per se;* in so far as there can be said to be a regulation of any "steady states" in the composition of the blood (and tissues), our attention may be directed toward two features only: a, the total water content (probably, we suggest, in reference to the tissue proteins) and b, the concentration of salt (or of particular salts). If these two states are physiologically satisfied, then the total osmotic pressure may have, in principle, any value whatever. With special reference, then, to the exchange of water between

the organism and its environment, we may observe what happens to the organism that moves from fresh water to sea water.

In fresh water, the circumstance that the *milieu intérieur* is hypertonic to the *milieu extérieur* causes the organism to abstract water from the latter, this absorption occurring mainly, though not necessarily, by way of the oral membranes and perhaps, to some extent, by the gills. This constant absorption of water is offset by the constant excretion of urine which is hypotonic to the blood. The fact that simultaneously salts derived from food, along with metabolic products formed within the body, are being excreted in this urine is entirely secondary to the water exchange itself.

When the organism moves into salt water it finds itself faced with a tendency to lose water from its body; since it cannot excrete a urine which is hypertonic to its blood, and since all that it has available for urine formation is a solution that is already hypertonic to the blood, it is threatened both with bodily dehydration and the complete arrest of urine formation.

Two methods have been found to meet this problem, as exemplified by the teleosts on the one hand, the elasmobranchs on the other:

The teleost, when it moves into salt water, maintains the water content of its body by "drinking" sea water. The bulk of the hypertonic sea water and its contained salts are absorbed from the gastro-intestinal tract; the salt is excreted in a concentrated state by the extrarenal route and water is left "osmotically free" for hypotonic urine formation.

The elasmobranch in salt water conserves its urea by reabsorbing this substance from the urine and by developing branchial and oral membranes which are relatively impermeable to it; the concentration of urea in the blood rises until the *total osmotic pressure* of the *milieu intérieur* again exceeds that of the *milieu extérieur*, at which time water again moves into the body in natural response to the osmotic gradient and urine formation is restored (though at a lower rate). With the resumption of urine formation urea is again excreted from the body and a further rise in osmotic pressure by the further accumulation of urea tends to be checked. Thus the water balance and the formation of urine in the marine elasmobranch become "linked" with the renal excretion of urea because the excretion of urea determines the urea content, and hence the osmotic pressure, of the blood; and this in turn determines the rate of water absorption. Hence the "regulation" of the water content of the body as well as of the rate of urine flow depend upon the regulation of urea excretion—that is, upon the quantity of urea reabsorbed by the kidney tubules relative to the composition of the blood. In a dehydrated or "thirsty" elasmobranch the arrest of urine excretion leads automatically, by a rise in blood urea and an increase in water absorption, to a restoration of urine excretion; the animal is never required to "drink" sea water, though it may do so incidental to feeding.

The fact that the oral membranes, the gills and the gastro-intestinal tract are "leaking" urea from the body does not alter the significance of this cycle; it only requires a more complete conservation of urea by the kidneys. It is in line with this economy that the elasmobranch has developed branchial and oral membranes which are relatively impermeable to urea and which therefore reduce this leakage to a minimum.

The regulation of the salt content of the blood, both in the teleost and the elasmobranch, is a process which appears to be essentially independent of this fundamental water cycle. With water always available, salt and metabolic products can be excreted in the hypotonic urine without difficulty.

But the elasmobranch also excretes salt extrarenally; and we may inquire, is this extrarenal excretion in any measure "hypertonic," as in the case of the teleost, and therefore of a nature to liberate water for the formation of urine? We cannot answer this question directly from the nature of the urine because the urea cycle in itself furnishes water for the formation of a hypotonic urine. But as indirect evidence, we call attention to the Mg:Cl ratio in the urine. For it is typical of marine elasmobranchs that, as compared to sea water itself, there is relatively more Mg in the urine than there is Cl. Since considerably more Cl is absorbed than Mg (from the intestine), this fact shows that Cl has been excreted by an extrarenal route; and the excretion of Cl from a level of 270 mM. per liter in the blood to a level of 500 mM. per liter in sea water represents a process of osmotic work, and, in principle, a process that would leave water behind in the blood, "osmotically free" for the formation of a dilute urine. If water is left "osmotically free" in the marine teleost by the extrarenal excretion of chloride, it would also be left free in the elasmobranch.

The extrarenal excretion of NaCl and KCl is, apparently, a very primitive process, for it is common to the fresh and salt water elasmobranchs as well as the fresh and salt water teleosts. It may represent a primitive mode of regulating the specific salt composition of the blood, or it may be a primitive mode of regulating the water content (as in the teleosts). As we have previously pointed out (Smith, 1930) the branchial membranes are so organized as to maintain a high concentration of salt against pure water outside; when, in sea water, these membranes continue to excrete salt into the surrounding medium, the organism is only carrying on qualitatively in the mode of its fresh water ancestors, and all that is required is that the branchial level be elevated with respect to the blood. It is interesting to note, in this connection, that the osmotic pressure of many invertebrates is higher than that of sea water, and that osmotic regulation is effected by extrarenal means (Schlieper, 1930).

The extrarenal excretion of urea and ammonia (Smith, 1929b) are probably incidental and unrelated phenomena; the urea excretion appears to

be a process of passive diffu ion, from a low blood level in the teleosts and a high blood level in the elasmobranchs, and the excretion of ammonia is probably related to acid-base equilibrium.

We are in the end, then, reduced to the conclusion that the elasmobranch resembles the teleost not only in its ability to excrete a hypotonic urine and its inability to excrete a hypertonic urine, but also in its ability to excrete salts extrarenally, even in sea water. But the elasmobranch has added to this common and presumably primitive cycle, the renal conservation of urea which, by raising the osmotic pressure of its blood, enables it to absorb water directly and relieves it of the need to drink sea water continuously. There is nothing to tell us, however, whether the urea mechanism of the elasmobranchs is a unique specialization peculiar to them, whether it represents an exaggeration of a mechanism shared by the vertebrates generally, or whether it represents a mechanism that was once important in ancestral forms but is now decadent in all except this order.

The significance of water regulation in the elasmobranch and the teleost in relation to the evolutionary history of the vertebrates will be discussed at another time.

SUMMARY

An examination of fresh water and marine elasmobranchs leads us to conclude that the uremia which characterizes this sub-order of fishes results from the renal conservation of urea on the one hand and the relatively low permeability of the branchial and oral membranes to this substance on the other.

Evidence is presented to show that this uremia, by raising the osmotic pressure of the blood, enables the marine elasmobranch to abstract water from its environment in the same way as does a fish in fresh-water, and to excrete a urine which is isotonic or hypotonic to the blood in compliance with the osmotic limitations of the fish kidney.

This special function of urea in these fishes appears to be a feature which is added to the primitive method, shared by them and the teleosts, whereby isotonic or hypotonic urine is obtained by absorbing sea water from the gastro-intestinal tract and excreting the salt by an extrarenal route. The urea mechanism of the elasmobranchs frees them from the continuous drinking of sea water.

In both groups it appears that the fundamental features which are physiologically regulated to a "steady state" are, first, the water content of the body relative to some as yet obscure reference—possibly the body proteins; and second, the specific salt content of the organism. The total osmotic pressure and the urea content of the body fluids are merely incidental to these fundamental features and have no homoiostatic significance *per se*.

We are indebted to the John Simon Guggenheim Memorial Foundation for a grant enabling us to examine fresh-water elasmobranchs in Siam and Malaya, the results of which work are in part incorporated in this paper.

We are also indebted to Mr. C. M. Breder of the New York Aquarium for coöperation over a period of several years in this problem, and to Mr. Nicholas Faranacci for technical assistance in many of the chemical determinations.

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THE EFFECT OF AGE, PREGNANCY AND LACTATION ON THE HEMOGLOBIN OF THE ALBINO RAT

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It is universally recognized that in any nutrition research work with animals normal standards are necessary for comparison. In 1926 at which time the studies in nutritional anemia were started in this laboratory, standard figures for the normal hemoglobin of the albino rat were meager (Donaldson, 1924; Williamson, 1926). Furthermore, the difference in methods of determination and the possible variation in animal strain made the reported normals inapplicable as criteria for our work. Early in 1929 a study of the hemoglobin of the stock animals of this colony was undertaken. In the course of this work it was noted that an anemia developed during gestation and parturition and that immediate recovery took place during lactation and the weeks following. The phenomena seemed to warrant further study. While these problems were being investigated two reports bearing on the subject were published by other workers (Sure, 1929; Wills, 1930). However, there was not complete agreement between either of these reports and our results. Therefore, we wish to present data from this laboratory on the normal hemoglobin of the stock colony and the temporary anemia, associated with pregnancy.

A. HEMOGLOBIN VARIATIONS IN STOCK RATS AT DIFFERENT AGES. *Animals.* Sixty-two albino rats from the stock colony were chosen for this study (34 females, 28 males). Observations on eleven of this group were begun at 23 days of age. The age of the others varied between 83 and 518 days. The young males were observed for 36 weeks, i.e., until 272 days of age. The young females were subjects throughout their life span including several periods of pregnancy. For the group of young animals hemoglobin determinations were made weekly, for the older stock animals biweekly for periods of 10 to 12 weeks.

Ration. The stock ration used consisted of:

	per cent		*per cent*
Dried whole milk	20	Dried whole wheat bread	42
Rolled oats	10	Wheat germ	2
Peanut meal	12	Yeast	0.5
Yellow corn (ground)	10	Salt	0.5
Dried celery tops	3		

Fresh greens were fed about five times a week. The adequacy of this ration has been demonstrated by five years successful use indicated by a high normal growth curve and a splendid reproductive record (Mitchell, 1930).

Method of hemoglobin determinations. The technique employed was essentially similar to that used in previous work (Mitchell and Miller, 1929) i.e., the method of Newcomer (1919) using a Bausch and Lomb hemoglobinometer with a blue filter in the eye piece. Details of the method as modified by us and the special precautions emphasized are described in another paper (Mitchell and Miller, 1931).

Results. The young animals on stock ration showed a rapid rise in hemoglobin values during the first four weeks of observation, the average

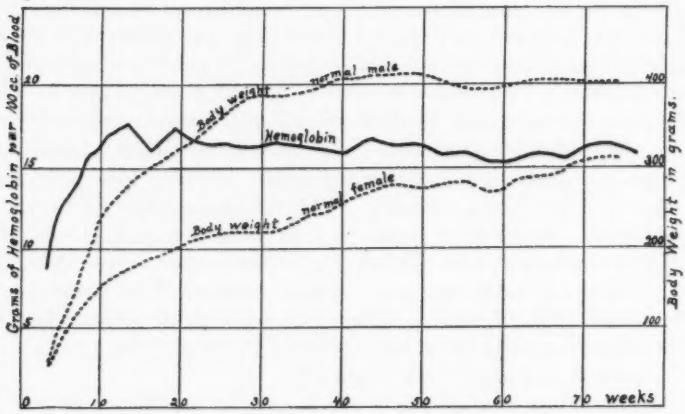

Chart 1. Average hemoglobin of rats on an adequate stock ration as related to age and growth.

values for these rats at 23 days of age being 8.9 grams per 100 cc. of blood, at 30 days 12.0 grams. It is interesting in this connection that experimental rats which had never received anything but milk showed at 30 days only 8 to 9 grams of hemoglobin decreasing to extreme anemic levels by 50 days. On the stock ration, however, the hemoglobin concentration increased rapidly reaching 15.6 grams at 50 days.

The average hemoglobin values for adult rats were arrived at by grouping the data according to the age of the animals, then averaging the weekly values of three week periods. Such values were considered the average for the mean week. In this way, each average represented 13 to 31 determinations. (See chart 1.)

In the above manner, it was found that within certain limits the hemo-

globin concentration of the adult rat (51–518 days of age) maintained a distinct plateau. The average was 16.2 grams of hemoglobin per 100 cc. of blood with a variation between 15.3 and 16.6, except for two instances which were higher. At the 12th and 18th weeks the average hemoglobin values rose to 17.5 and 17.2 grams, respectively. At least 31 determinations were used in obtaining each of these latter figures. By grouping these data according to sex, no significant difference was noted, thus one normal curve for both sexes is given in chart 1.

Discussion. These hemoglobin figures, both for the rapidly growing and for the mature rat, are consistently higher than those reported by most other workers. The values given by Sure and co-workers for rats 23 days of age are 1.4 to 3.7 grams lower than our observations for rats of the same age and their values for rats 49 to 53 days of age are 2.0 to 5.0 grams lower than our observations for animals 50 days of age. For the mature rat the reported maxima in hemoglobin values range from 14.0 to 15.5 grams per 100 cc. of blood. Only the highest of these figures is in the lower portion of the plateau area that is described in this report. The 15.5 gram maximum reported by Williamson and Ets (1926) occurs the 150th day, whereas the value of 15.0 grams given by Sure and co-workers (1929) occurs the 270th day. All of these values are in marked contrast to the average hemoglobin value of 15.6 grams shown by our rats at 51 days and to the plateau average of 16.2 grams per 100 cc. maintained thereafter.

These differences might in part be explained on the basis of the instrument used and the method of determination. Sure *et al.* (1929) are the only group of workers that have employed presumably the same method of hemoglobin determinations in establishing a normal for a stock colony. However the shape of our curve is significantly different. The maximum is attained much earlier than in the preceding citations and the subsequent variations from the average are less. The maximum hemoglobin concentration is reached during the rapid growth period.

Moreover, the question of heredity and diet might be considered as factors. May not the continual inbreeding of rats on the same ration for a period of five years be capable of producing a difference in hemoglobin concentration and a difference in the relation of hemoglobin values to age as well as a difference in growth? It would be interesting to compare the results of several laboratories in this respect.

B. Hemoglobin variations during pregnancy and lactation. *Animals.* Data were collected on 145 pregnancies in fifty-one female rats. Thirty-two of these animals were under observation before the first mating. The experimental period for the major portion of the group covered three consecutive reproductive cycles. However, for eleven animals it was extended throughout the fourth or fifth pregnancy. Only five of the 145 litters numbered less than six young to a litter and the average was 8.2.

The litters were weaned at 21 days of age. The female breeders were then allowed to rest two and one-half to three weeks before the next mating.

Ration supplements. At the time of isolation, two or three days before parturition, the animals were given milk *ad libitum* and one teaspoon of wheat germ daily as supplements to the stock ration previously described. These supplements were continued throughout the period of lactation.

In an attempt to lessen the observed anemia of pregnancy an additional mineral supplement of iron, copper and manganese sulphates was used (0.5 mgm. of Fe, 0.05 mgm. of Cu, and 0.1 mgm. of Mn). This combination of minerals was thought at that time to be the most efficacious for the relief of nutritional anemia in the growing rat. We now realize that the manganese may have had little significance with reference to hemoglobin regeneration. These salts in solution were administered daily from the time of mating until the young were two weeks of age.

A vitamin B complex supplement was used in a similar manner. Sure and co-workers had reported that fortification of the ration with vitamin B complex seemed to be effective in the relief of the anemia associated with pregnancy (Sure, 1929). A yeast extract,[1] which had been found to be very rich in both vitamins B and G (unpublished data) was fed in liberal amount,—0.5 gram daily.

Neither of these dietary supplements was limited exclusively to early or late pregnancies, but was so distributed that pregnancies of all orders, except the first, were observed with both supplements and with none. The milk and wheat germ were always given as described above in addition to the mineral or the vitamin supplement.

Hemoglobin determinations were made weekly in all but a few of the very earliest observations when they were made biweekly.

Results. In all cases of pregnancy observed, a developing anemia became evident within the first five days of gestation and increased as the period of gestation advanced. An average total drop of more than three grams in hemoglobin concentration occurred as shown in chart 2, the low point being within the three day period following parturition. Of the 145 pregnancies observed there were only 12 cases in which some degree of anemia failed to develop prior to parturition. But these cases did show a lowered concentration of hemoglobin during the three days following parturition. The lack of conformity of these few was assumed to be a natural variation in rats as it could not be correlated with the size or order of the litter, with the dietary supplements, or with the hemoglobin concentration prior to or at the time of breeding. In 92 per cent of the pregnancies observed an anemia developed before parturition.

A study of chart 2 reveals further that the rise in hemoglobin concentration after parturition was as striking as its previous fall. During the first

[1] Savita,—Battle Creek Food Co.

14 days of this period the hemoglobin values rose from 12.8–13.0 grams to 15.1–16.2 grams per 100 cc. of blood. Then during the seven days just prior to weaning the young the gain was retarded, regardless of any change in ration at that time. Approximately the same rate of increase as that of the first 14 days was resumed during the nine days following the period of lactation bringing the hemoglobin level higher than the normal plateau described in part A. This rise was followed by a decline to within normal limits. Only six failed to show this characteristic recovery after parturition. Five of these had shown a slight gain just prior to parturition and maintained an approximate level during the lactation period.

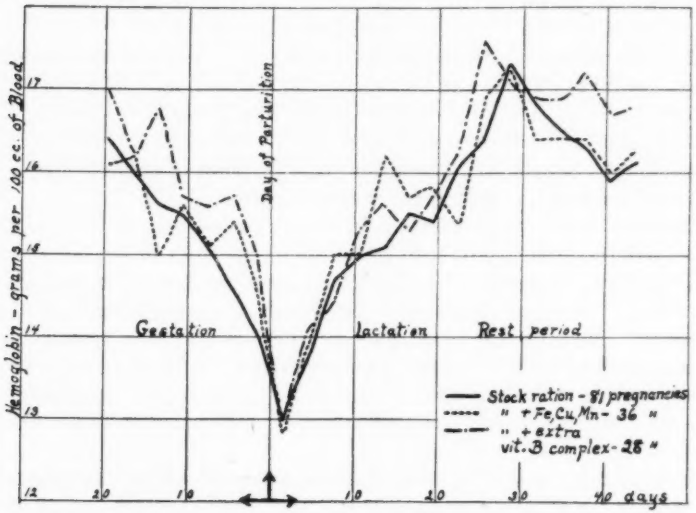

Chart 2. Hemoglobin averages of female rats during the reproductive cycle. The weekly hemoglobin values falling within each three day period before and after parturition were averaged and plotted as the mean for the period.

Neither of the dietary supplements, the mineral mixture of iron, copper and manganese, nor the vitamin B complex exerted any apparent influence in preventing the anemia of pregnancy just described or in hastening the subsequent recovery. The marked irregularities which occurred in the hemoglobin levels of the supplemented groups may or may not have significance.

These observations on hemoglobin of the rat during pregnancy were studied in relation to the age of the mother, to size and order of the litters as well as to the dietary supplements. None of these factors seemed to have much bearing on this physiological phenomenon.

Discussion. The development of an anemia in female rats preceding parturition, a further drop during three days following and the subsequent recovery have in part been noted by other workers, but continuous observation throughout a series of pregnancies has not, so far as we know, been reported in the literature. A slight drop in hemoglobin concentration in rats 24 hours before delivery was noted by Wills and Mehta (1930), but it was not considered as being significant. Likewise Sure and co-workers (1929) observed an anemia the day of parturition, and attributed it to hemorrhage because of its temporary character. With their group of animals this anemia apparently did not develop when the ration was reenforced with vitamin B complex. In the 145 pregnancies observed in this study the anemia is progressive during the period of gestation and is not a condition due alone to hemorrhage at parturition.

There are frequent references in the literature to a similar condition during human pregnancy (First and Goldstein, 1930; Bland and Goldstein, 1929; Galloway, 1929). First and Goldstein offer four possible explanations: *a*, withdrawal of iron from maternal corpuscles by the fetus; *b*, maternal blood destruction by a syncytial hemolysin; *c*, occurrence of chloranemia, or *d*, relative deficiency due to increase in blood volume.

Nicholas' (1928) observations lend weight to the first of these suggestions. He found hemoglobin in measurable quantities in the fetus before the 14th day of the gestation period but the greatest increase in hemoglobin concentration came between the 14 and 17th days, the early part of the third trimester. Our curves in chart 2 show the greatest drop in hemoglobin of the mother during the third trimester regardless of supplement, the period corresponding to that of greatest increase in the fetus. Therefore this demand of the fetus probably accounts in part for the decrease in hemoglobin concentration of the mother.

Clinical evidence also points to the hemorrhage of delivery as responsible for the further drop in hemoglobin and erythrocytes which occurs in the mother during the 48 hours after the birth of the child. The progressive anemia during the 3 days following parturition in the rat correlates well with these observations on the human.

The spontaneous recovery from anemia after childbirth in humans also has a counterpart in the rat. The hemoglobin concentration increases rapidly during lactation and the following week reaching a temporary level higher than the normal plateau to which it drops within a few days. This increase has been observed irrespective of the dietary reinforcements which have been added.

The progressive character of these changes associated with the periods of gestation and lactation which occur in spite of the dietary supplements employed seems to indicate that the apparent anemia is a physiological condition dependent on pregnancy *per se*, as suggested by Bland and Goldstein (1929), and not a pathological phenomenon.

SUMMARY

1. The hemoglobin of young rats from our stock colony increased from 8.9 to 15.6 grams during the period from 23 to 50 days of age.

2. The hemoglobin for adults in the stock colony was found to average 16.2 grams fluctuating between 15.3 and 16.6 grams.

3. The development of an anemia in female rats preceding parturition, and progressing for three days following with a subsequent spontaneous recovery has been observed in at least 92 per cent of the 145 cases investigated.

4. A mineral supplement in the form of iron, copper and manganese salts and additional vitamin B complex in the form of a yeast extract were added to the rations of experimental females during various reproductive cycles. Neither of these dietary reenforcements exerted any apparent influence in preventing the anemia of pregnancy.

5. The character of the hemoglobin changes associated with pregnancy seems to indicate that this apparent anemia is a physiological condition and not a pathological phenomenon. Clinical reports of anemia associated with human pregnancy offer an interesting analogy to these observations in the rat.

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THE TIME RELATIONS OF THE ELECTRICAL AND MECHANICAL RESPONSE OF HEART MUSCLE

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This series of observations, while made more or less incidentally during another investigation, have a direct bearing upon Einthoven's hypothesis that the latent period of muscle contraction is apparent rather than fundamental and that the electrical and mechanical responses are actually simultaneous. Specifically, they concern the work of de Jongh (1926), who publishes graphic records taken with Einthoven's Saitenmyographion from the excised frog's ventricle that are interpreted as confirming this hypothesis as applied to heart muscle.

APPARATUS. During a study on the relation of the metallic ions of the blood to the origin of the cardiac impulse, need arose for recording the mechanical beat along with the electrical response: Mines' (1913) method of observing the beat visually was found inadequate, as were his torsion-lever and the "inscripteur a corde" used by Einthoven and Hugenholtz (1921). The following instrumentation was accordingly developed. Though different in appearance, it is in principle a modification of Einthoven's Saitenmyographion (or Snaarmyograph), the most sensitive myograph available and said to possess the same degree of accuracy as the string galvanometer (Fulton, 1926). Whereas the cost of Einthoven's instrument is over $1000 the present recorder can be largely put together of standard laboratory materials.

The essential part is the moving-element (fig. 1), which consists of a "triangle" of fine silver wire, L, soldered to the center of a manganin torsion-wire, A; the ends of the manganin wire are soldered into the tips of rods B and C. The triangle L is right-angled, with the vertical arm extending for some distance beyond the vertex; its base rests upon the excised heart, and with each beat of the contracting tissue the triangle rotates as a unit upon the torsion-wire as an axis. The resulting displacement of its vertical arm is highly magnified by a horizontally mounted microscope M, which projects a shadow of the arm upon the camera-slit. The source of illumination is a concentrated mazda filament.

Rotation of rods B and C by means of their milled shoulders J applies a

torque to the manganin wire, lowering the base of the triangle upon the tissue. The torsion is usually carried beyond the point necessary for mere contact of triangle and tissue, the triangle exerting a positive downward pressure. This gets at contractions of deep-seated fibres too distant to affect the surface of the muscle and facilitates detecting the inception of the mechanical changes.

The degree of optical magnification is controlled by varying the size of the ocular and the distance of the whole apparatus from the camera. A magnification range of 100–1400 has been used. Since the highly magnified arm-shadow becomes too thick for photographic purposes, only one (sharply focused) edge is used in the records.

To enhance the efficiency, the moving-parts were made as small and light as possible, and most of the magnification was obtained by optical rather

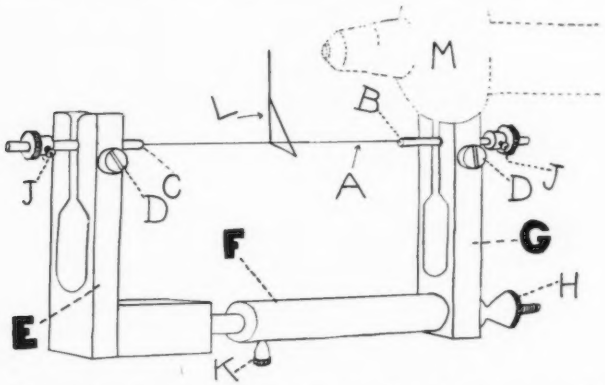

Fig. 1. The moving element of the myograph and its adjacent parts. Description in text.

than mechanical means. The torsion-wire was 0.1 mm. in diameter, $\frac{1}{5}$ the thickness used by Einthoven, and the silver wire finally chosen for the triangle was 0.008 inch,—considerably thinner, judging from published diagrams, than the material of the Saitenmyograph. The glass-tubing supporting Einthoven's "Ansatzstück" is eliminated, as are the 1.5 cm. ring for the quartz-fibre, and the cathode with its attached wire,—all part of his moving-system. The present design would thus seem to be of superior sensitivity. Since, however, de Jongh gives no figures on either the sensitivity or frequency characteristics of the Saitenmyograph, his experiment was repeated to provide a pragmatic comparison of the two instruments and to indicate whether our myograph could reliably be used in the larger study above mentioned.

Registration. Our method differed somewhat from de Jongh's. He

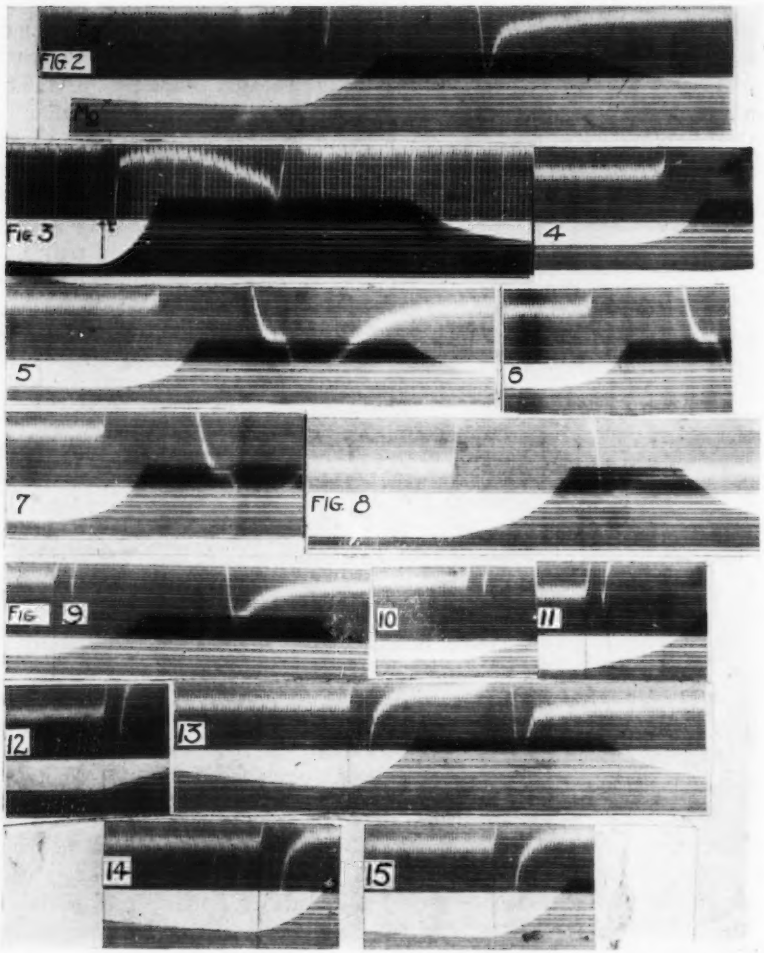

Fig. 2. The most frequent type of curve; the initial deflection in the electrogram, *Eg*, definitely precedes that in the mechanogram, *Mg*. Film-speed 108 mm. per second. Galvanometer-leads: proximal (i.e., triangle) on ventricular wall about 1 mm. from cut surface; distal on injured apex. Figures 3 to 8—showing that the artefactual precedence of *Mg* may be a function of the pressure of the recorder as well as of the location of the galvanometer leads. In figures 4 to 7 the time interval between *Mg* and *Eg* increases progressively with increase in triangular pressure. Galvanometer leads: proximal as above; distal on uninjured apex. Time lines 0.02 second. Period of galvanometer string 0.004 second or less. Figures 9 to 15—leads as in figure 2. Curves *13* and *15* are approximately simultaneous.

placed the receiving element of the Saitenmyograph "on the ventricle," one galvanometer lead on the injured apex and the other on the cut-surface (rim of the spongework-core). Believing that the electrical and mechanical responses should be registered from the same bit of muscle-tissue, we constructed our triangle of pure silver wire to serve both as a non-polarizable lead and as the mechanical lever. The ventricle, cut away at the coronary sulcus, was laid on its side on a slow-motion elevating-stand. The base of the triangle was electrolytically coated with AgCl and placed upon the ventricular wall parallel to the cut-surface and very close to its edge. The distal electrode, either of the Zn-ZnSO4 or the AgCl type, was fixed upon the apex of the ventricle.

A. *Frog records.* Figures 2 to 15 show, among other things, that all three types of curve published by de Jongh are procurable with the present recorder: those in which 1, the electrogram, *Eg*, precedes the mechanogram *Mg*; 2, the *Mg* precedes the *Eg*, and 3, *Eg* and *Mg* are approximately synchronous.

1. The type most easily obtained and most numerous statistically was that in which *Eg* preceded *Mg* (fig. 2). The upper curve is the *Eg*, the lower the *Mg*. This record was taken from the heart used in figures 9 to 15 below and under the same conditions. The tissue beneath the distal lead was injured.

2. Figures 3 to 7 present one of two types of records in which *Mg* precedes *Eg*. Here, the distal lead was on the *uninjured* apex of the ventricle. The film speed was 108 mm. per second (de Jongh's was 25 mm.). In figure 3, from the interval marked *t* the natural period of the galvanometer string can be seen to have been 0.004 second, if not less. Assuming that the initial deflection in the *Eg* started at the time-line indicated by the arrow, the *Mg* had already begun to rise. Figures 4 to 7 were taken at progressively higher torsions of the manganin wire (after the heart had again been moistened with Ringer's[1]), and show correspondingly longer intervals of anticipation of the *Mg*. The location of the leads was the same, the only changing factor being pressure of triangle on tissue. In curves 4, 5, and 6 the sharp *R* wave is definitely preceded by a deflection which rises more slowly from the zero-level of the curve; the *Mg* precedes even this slower deflection.

This result is an artefact, as de Jongh indicates, and betokens delayed detection of the electrical response due to the disposition of the leads. Lewis (1916) has shown that in the frog's ventricle the excitation wave is transmitted through the deeply-seated sponge-work, first reaching the surface in the central parts, and that there may be simultaneous propagation

[1] Remoistening was necessary to bring back the sharp spike-wave without which the initial rise in the *Eg* could not readily be determined. In figures 4 to 7, when the spike-wave reappeared, the *Eg* was of different wave-form.

of the wave to a number of basal and apical points. The resulting temporary isoelectricity of the surface leads might well produce the delay in question. This is further indicated by the presence of the slower deflections mentioned above, probably of "extrinsic" origin.

After curve 7, a new series was taken under a range of tensions from very low to very high, to ascertain whether, despite the leaving intact of the apical tissue, the tension factor alone could bring about a simultaneous *Eg* and *Mg*. Figure 8 is the curve most nearly simultaneous. The *Mg* still precedes, though by a very much smaller interval. All of these curves indicate that, in addition to the disposition of leads, the torsion on the manganin wire is a determining factor in the time-relationships appearing in the records.

Figures 9 to 12 show the other type of curve in which *Mg* precedes *Eg*, the tissue beneath the distal contact having been seared with a heated metal rod.[2] The interval is small but noticeable.

3. Figures 13, 14 and 15 illustrate the type in which *Eg* and *Mg* are approximately simultaneous. Of his published simultaneous curve on the frog's ventricle (Abbildung 7), de Jongh writes, "The Eg and Mg begin synchronously, indeed the Mg in our curve perhaps even precedes the Eg somewhat." We likewise found it difficult to affirm synchronism with certainty, due to the "spread-out" curve caused by our more rapid film-speed and to the adventitious vibration of the string (cf. footnote 3); even examination of the records with a Lucas comparator failed to be of much service. For the purpose however of comparing our instrument with the Saitenmyograph, it was sufficient to reproduce curves as close to simultaneity as Abbildung 7. Figures 13 and 15 meet this requirement; and are of added significance because in de Jongh's opinion our location of the galvanometer leads increases the difficulty of obtaining simultaneity.

Figures 13 to 15 were taken from the same heart used in figures 9 to 12, with the same disposition of the leads but at another triangular pressure. Figure 2 was clipped from this last series, appearing spontaneously among curves which were simultaneous or with *Mg* preceding.[3]

[2] Despite this injury, the wave-form is not monophasic. For a detailed consideration of similarly "abnormal" wave-forms in frog electrograms, cf. Samojloff (1910) and Mines (1913).

[3] *Further points of technique.* Since the horizontal lines in the photographs interfere with accurate determination of the initial rise of the *Mg*, the etched grating should be removed from behind the camera-lens.

The periodic motion of the galvanometer curve was found to be due to 1, induction from vibrating wires which fed the myograph lamp; fastening them reduced the unsteadiness; and 2, mechanical vibration of parts of the set-up, communicated to the triangle and resulting in a varying electrical contact with the tissue. This was traced to the camera and largely overcome by screwing and clamping all parts to the heavy oak tripod supporting them. If this is inadequate elsewhere, the vibration-eliminating support described by Fulton (1925) may have to be provided.

DISCUSSION OF FROG RECORDS. While the three types of curves obtained by de Jongh are thus reproducible, it is difficult to agree altogether with his conclusions.

1. One reason is the comparative paucity of the simultaneous curves. In the vast majority taken with our myograph either the *Eg* or *Mg* preceded. That de Jongh's simultaneous curves were likewise not very numerous may perhaps be inferred from the fact that even in the one curve on the frog's ventricle published as proof of simultaneity there is some doubt about the synchronism. Examination of this Abbildung 7 with a reading glass shows the *Mg* actually precedes. Unfortunately, no additional simultaneous curve is presented nor is the numerical frequency of the simultaneity finding stated.

2. This statistical infrequency gathers further significance from a consideration of the factor of "optimum pressure" which is a *sine qua non* to the attainment of simultaneous records. De Jongh remarks that too much as well as too little pressure may prevent the simultaneity from appearing in the record; "the optimum degree of pressure must be ascertained empirically in the course of the experiment itself" (p. 227). This empirical determination was not a simple matter, we found; it involved the taking of many records at all possible pressures, that pressure being taken as optimum which, in the developed photographs, gave the most nearly simultaneous curves. Frequently, of a dozen or so consecutive beats recorded under the same torsion, only one or two are simultaneous or nearly so.[4] It

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A liquid or wick electrode in place of the triangle lead gives greater stability of the *Eg* and also greater galvanometric sensitivity, since the triangle tends to dry at its point of contact with the tissue and hence increases in resistance. The triangle, when not essential as a galvanometer lead, can be made of duralumin instead of silver, the greater rigidity and lightness of duralumin yielding a more sensitive system, but soldering it to the manganin wire is difficult. Platinum-iridium has even greater stiffness and is more easily soldered.

Since the moving-system is conveniently removable, several interchangeable elements of varying sensitivity and frequency characteristics can be prepared for different experimental applications. The characteristics will vary with the size of triangle, diameter and length of manganin wire and only within restricted limits with lateral tension: High vibration frequency necessitates a shorter and thicker torsion-wire, while high sensitivity goes with a longer and thinner wire, thinness being considerably more important than length. We have used wires as long as 10 cm. and some as low as 0.02 mm. in diameter.

[4] One record (hereafter example A) starts with *Eg* preceding but the interval is decreased with each beat to approximate simultaneity in beats 7 and 8. Occasionally, the temporal relationship between *Eg* and *Mg* is actually reversed: thus, (example B) in one series beats 1 to 5 show *Mg* preceding, 6 shows *Eg* preceding, 7 and 8 show *Mg* again preceding.

Since there was no change in the torsion during a series, the net tension at any given beat is possibly determined by the degree of tonus or internal tension of the

is this more or less fortuitous aspect of the simultaneity finding that gives one pause.

De Jongh would probably regard those curves in which *Eg* precedes a result of unfavorable conditions of mechanical registration, e.g., non-optimum pressure of triangle; while the precedence of the *Mg* he might consider a result of our disposition of the leads: For, assuming that his myograph and electrodes were at optimum location and the wave of excitation started just beneath his proximal lead, this wave would have to traverse a minimal distance of 2 mm. to reach the tissue beneath our triangle, causing a delay of 50 σ in our electrical registration (taking conduction-time in ventricular muscle as 400 mm. per sec.). Hence precedence of the *Mg*. This fails to explain, however, why figures 13 and 15 are approximately simultaneous even with our non-optimum disposition of the leads (unless it be said the 2 mm. distance was reduced by the compressing effect of the triangle).

Such interpretations are not completely satisfying, however. If, as de Jongh assumes, both the too large and too small tension give a spurious result caused by faulty conditions of recording what objective justification is there for thinking the simultaneity result any more reliable? Ordinarily, in view of its statistical infrequency, one would tend to discount the simultaneity finding and attribute it as well to the particular pressure conditions which chanced to yield it. As far as present experimental evidence goes, *the only criterion of the reliability of de Jongh's optimum pressure seems to be that it happens to yield the simultaneity called for by Einthoven's hypothesis. In reality, after all presupposition is ruled out, the results on the frog's ventricle could be interpreted as evidence either for or against this hypothesis.*

3. De Jongh's method of "leading off" likewise calls for further consideration. With optimum myographic pressure attained, he feels sure of earliest registration of the mechanical response. He then seeks earliest

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heart-muscle as well as by the torsional setting,—the net tension being a resultant of the two. In the various diastoles the extent of relaxation may vary, and the elevation of the triangle accommodates itself to resulting differences in the level of the heart tissue. This is perhaps borne out by the frequent variation in the height of the zero-levels from which the systolic curves rise. Thus, in certain records, there is a decrease with each beat: in one case the *Eg* preceded *Mg* by 0.04 second in the first beat, this interval increasing with each beat to 0.08 second in the fourth beat; the corresponding heights of the zero-levels from the lower edge of the film were 17.5, 19, 21, and 22.5 mm., the net tension thus decreasing with each beat. In example A above, there was an increase in net tension with each beat. In example B, the zero-levels in millimeters were 6, 8, 9, 9.5, 10, 11, 12, 12; the *Mg* preceded at 6–10 mm., the *Eg* preceded at 11 mm., and the *Mg* again preceded at 12 mm. If progressive decrease in tension tends to push the *Eg* ahead, as at 11 mm., the *Eg* should be still further ahead at 12 mm.; instead, it again falls behind the *Mg*. Apparently, the factor of tension is not the whole story. This whole matter needs further study.

registration of the electrogram. Accordingly, with the myograph remaining on the ventricular wall and the distal lead on the injured apex, he shifts the proximal contact about and finds the *Eg* appearing earliest with this lead on the cut-surface,—as in Abbildung 7. This shifting of the proximal lead independently of the myographic contact seems questionable, since the excitation wave might affect the cut-surface electrode before reaching the tissue beneath the myograph (cf. Lewis, 1916). Since even with this lead, the *Mg* precedes slightly, it seems possible that what de Jongh had here was a series of curves in which *Mg* for some reason preceded *Eg*, which interval he shortened by placing the proximal lead on the cut-surface. With this lead on the tissue under the myograph, there might not have been the close approach to simultaneity of Abbildung 7. Since the moment of appearance of *Eg* can thus be varied with the location of the proximal lead, it would seem more accurate to lead off both the electrical and mechanical responses from the same bit of tissue, as is done when the triangle is used as a non-polarizable electrode.

The only source of error here arises where the excitation wave begins proximal to the triangular lead, thus introducing the possibility of "extrinsic" deflections interfering with exact determination of the time-relations. While these adventitious deflections can usually be distinguished from intrinsic ones, it was thought advisable to eliminate their occurrence if possible and, following a suggestion of Professor Howell, to substitute the isolated terrapin-sinus for the frog's ventricle. The terrapin-sinus consists of a single, fairly homogeneous layer of muscle tissue; the ventricle, however, contains a sponge-work core which contracts independently of the remaining muscle, so that a response recorded from the ventricular surface is a composite affair and accurate interpretation of the curves is sometimes difficult. Of this disadvantage, the sinus seems to be free.

B. *Records from the excised terrapin sinus.* After a rapid operation the excised sinus was at once perfused with Ringer's. The perfusion was stopped while the records were taken and resumed between records. A bit of adjacent tissue removed with the sinus was seared with a heated metal rod and the indifferent electrode placed upon it. The active electrode, i.e., the triangle, was placed at the head of the sinus near the inflow cannula. To secure "optimum pressure," a low degree of torsion on the manganin wire was first used and then increased by progressive increments, a few beats being recorded at each stage. The object was to run a single heart through all the tensions, observing the effect of passing through optimum tension on the temporal relation of *Eg* and *Mg*. The beats became enfeebled, however, and on no single heart could the series be completed. Some hearts were therefore started with a relatively high tension and continued on from that point. With the large number of observations from different hearts under a variety of tensions, the probability is that some curves must have been registered at "optimum" pressure.

In all, about 800 curves were recorded. Of these, approximately 100 were discarded because the absence of a sharp spike-wave made it impossible to determine the point of initial rise of *Eg*. This type of curve was taken to indicate deterioration of the excised tissue. In the remaining 710 curves there was not a single clear case of simultaneity, the *Eg* preceding in practically every instance. This seems to contradict de Jongh's results on the frog's ventricle and rat's heart. A possible source of error, however, is that the mechanogram was always taken on the collapsed sinus, and possibly, therefore, not under normal conditions. In addition, our myograph was designed primarily for high sensitivity rather than high natural frequency (to determine whether cardiac tissue was or was not contracting under given perfusion conditions), and although we believe its frequency characteristics compare favorably with those of the Saitenmyograph, no figures are available for a numerical comparison.

It is not urged that these results establish the temporal precedence of the electrical response. Their general trend, however, indicates that de Jongh's findings are not entirely conclusive and that the problem calls for further investigation.

SUMMARY. 1. An instrumentation for registering the mechanical and electrical responses of contracting muscle is described. Its advantages are: *a*, in sensitivity, it compares favorably with and is perhaps superior to Einthoven's Saitenmyographion, due largely to the greater delicacy of its moving system; *b*, the moving element is conveniently removable, so that interchangeable elements may be used for different experimental applications; *c*, it can be largely put together of standard parts; *d*, it is comparatively inexpensive. Its chief disadvantage is its susceptibility to adventitious mechanical vibration, necessitating careful mechanical shielding. De Jongh's study on the temporal relation of the electro- and mechanogram of the frog's ventricle was repeated, showing that this apparatus can register all types of curves obtained with the Saitenmyograph.

2. Implications of the records are that de Jongh's conclusion as to the simultaneity of mechano- and electrogram is not entirely convincing, due to 1, comparative infrequency of simultaneous curves; 2, lack of outside criteria of the reliability of "optimum pressure," and 3, his disposition of the galvanometer leads.

3. Of 710 curves from the excised terrapin sinus, there was not a single clear case of simultaneity, the *Eg* definitely preceding *Mg* in practically every instance.

CONCLUSION

These results point to the possibility that the simultaneity obtained by de Jongh may be fortuitous rather than fundamental in nature. The temporal relation between mechano- and electrogram thus remains an open question.

The writer desires to express his indebtedness and thanks to Prof. W. H. Howell for his advice and assistance in this work.

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A STUDY OF TENDONS, BONES, AND OTHER FORMS OF CONNECTIVE TISSUE BY MEANS OF X-RAY DIFFRACTION PATTERNS

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A number of German investigators have taken x-ray diffraction patterns of tendons and cartilage. They obtain substantially the same pattern from tendons and stretched gelatin, the first hydrolysis product of collagen.

Katz and Gerngross (1), using the K_α ray from copper ($\lambda = 1.54$ A. U.), obtained, for isoelectric gelatin, a broad amorphous ring (identity period 4 A. U.) and a sharp crystalline ring (identity period 2.7 A. U.). When stretched (100 per cent elongation) two spots appear close to the centre beam (10 A. U.) at right angles to the direction of stretching. The amorphous ring becomes an ellipse with a spacing of 3.9 A. U. in the stretching direction, and two diffuse spots appear on the inside of the ring at right angles to the stretching with a spacing of 5.0 A. U. The crystalline ring becomes two arcs in the direction of stretching (2.7 A. U.) (see fig. 1). Clark and Lanyon have confirmed these observations on isoelectric gelatin and give the identity periods as 4.2 and 2.8 A. U. Herzog and Jancke (2), also using the K_α line of copper, got the same diagram from dried tendon (fibrous collagen) that had been obtained from stretched isoelectric gelatin. In a subsequent paper Herzog and Gonell (3) investigated collagen from six different sources, the materials being dried, in some cases under tension, after having been treated with trypsin for 36 hours to remove other organic material They found that collagen from all sources gave the same diagram.

A number of investigators have found distinct crystalline patterns from bones and teeth. It has invariably been stated (4), (5), (6) that the solid inorganic phase in bone, dentine and dental enamel consists of crystalline apatite.

METHOD. The work of the German investigators has established the fact that collagen gives a definite x-ray diagram. As the material used was always dried, and usually treated with trypsin before drying, it was not known whether collagen, as it exists in the living tendon, gives a crystal pattern or not. Accordingly double pinhole x-ray diffraction patterns were taken with freshly excised material, kept alive with Ringer solution in

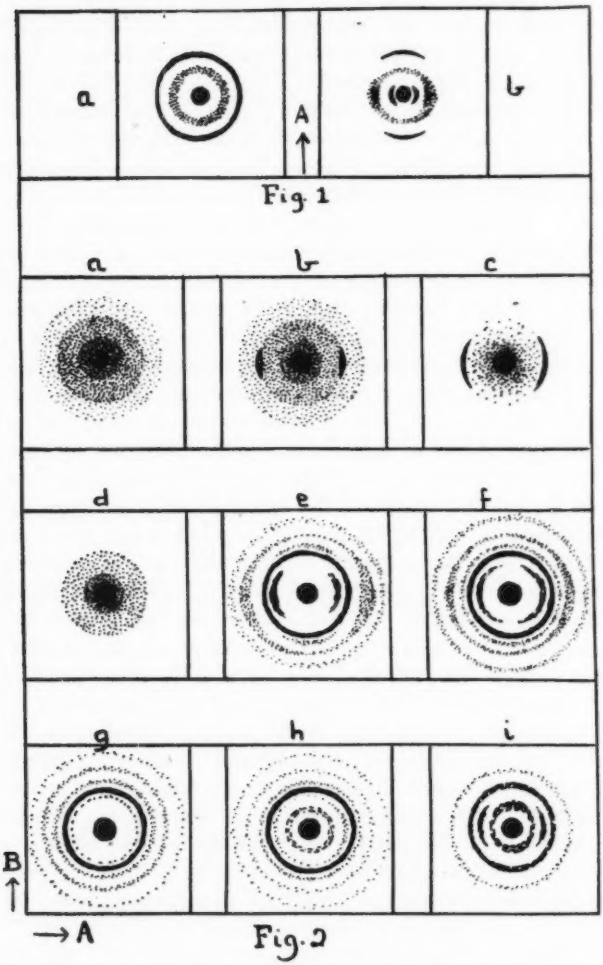

Fig. 1. Diagrams of x-ray diffraction patterns; *a*, isoelectric gelatin; *b*, stretched isoelectric gelatin; *A*, stretching direction.

Fig. 2. Diagrams of x-ray diffraction patterns; *a*, moist living tendon; *b*, moist living tendon stretched; *c*, tendon stretched and dried; *d*, costal cartilage; *e*, forearm bone rat; *f*, longitudinal section compact bone; *g*, cross section compact bone; *h*, dentine; *i*, enamel; *A*, stretching direction, direction of length of bones, direction of prisms in enamel.

a small moist chamber during the exposure time. As a copper tube was not available the K_α line of molybdenum was used ($\lambda = 0.712$ A. U.), which gave less good definition than the longer wavelength of copper. In no case was there any indication of the 10 A. U. spacing as this fell inside the centre spot and the amorphous ring became a diffuse haze in which there was no clear indication of arcs. The observations on collagen were therefore confined to the 2.8 crystalline ring which, though more diffuse than in the diagrams with the copper tube, was clearly discernible to the eye.

RESULTS. *Tendons.* The results on tendons were given in a previous paper (7). The diffraction pattern of a fresh unstretched tendon shows no crystal ring but only a diffuse zone indicating an amorphous or liquid crystal condition in collagen under normal conditions. The spacing associated with this zone is approximately 3.1 A. U., or 6.2 A. U. for double molecules. On drying the crystal ring appears and is associated with the spacing 2.8–2.9 A. U. The dehydration apparently decreases the molecular size and produces a change from amorphous, or liquid crystal, to crystal form. If a moist, living tendon is stretched the diffraction pattern shows spots at the edge of the amorphous zone, in the stretching direction, and if the tendon is dried in a stretched condition the pattern shows two clear arcs in the stretching direction (spacing 2.8–2.9 A. U.) (see fig. 2 *a*, *b*, *c*).

When a crystal pattern appears under tension it may be interpreted as due to the formation of rod-like crystallites or to the orientation of rod-shaped molecules. Trillat (8) believes that the appearance of arcs and spots in a pattern from a stretched substance indicates a passage from amorphous to crystalline material by gradual steps giving finally a pseudo-crystalline structure in which the molecules occupy the position they would in a crystal lattice but are kept from perfect crystal form by residual amorphous material. Another probable interpretation is a pseudo-crystalline structure in which rod-like crystallites are imbedded in residual amorphous material. It is possible therefore to interpret the diffraction patterns as evidence of the formation of crystalline collagen under tension.

As collagen occurs in many forms of connective tissue the work on tendons was extended to include a study of diffraction patterns from other forms of connective tissues, especially cartilage, bones and teeth.

Adipose tissue. Adipose tissue from under the skin of a rat gives a pattern with a clear crystalline ring (identity period 4.85 A. U.). This is probably one spacing of the fatty acids present, which solidify at room temperature.

Cartilage. The diffraction pattern from costal cartilage shows a very hazy zone with a 3.0 A. U. spacing when moist and a 2.8 A. U. spacing when dried. This zone is apparently due to the collagen present which is in amorphous condition (see fig. 2 *d*).

Bone. Bone consists of inorganic substances known as "bone earths"

(largely calcium phosphate with very little calcium carbonate) infiltrated into a ground substance of organic material called ossein. The chemistry of ossein is similar to that of collagen and the organic part of bone, like cartilage, contains mucoid and albumoid. There seems little doubt that ossein mucoid and albumoid are identical with the corresponding substances in cartilage. In fact bone has usually been thought of as a cartilage matrix with a deposition of inorganic material replacing portions of the matrix and the hardness of bone has been attributed to the presence of these inorganic constituents.

A study of the x-ray diagrams from bones suggests a somewhat different conception.

TABLE 1

(*Bones*)

	LONGITUDINAL SECTION		CROSS SECTION	
	Forearm bone rat	Compact bone (ulna dog)	Compact bone (ulna) spongy bone	Spacing A.U.
a	2 arcs direction of length			3.35
		Ring slight fibering	Very faint (doubtful)	3.5
b	Strong continuous ring	Strong continuous ring	Strong continuous ring	2.8
c	2 faint arcs perpendicular to length			2.2
		Faint ring	Faint ring	2.28
d	Ellipse with arcs in direction of length	Faint ring	Faint ring	1.7
e	(Probably 2nd order a)			1.6
f	Hazy ring (Probably 2nd order b)	Faint ring	Faint ring	1.4

Monochromatic pinhole diagrams from longitudinal sections and cross sections of compact and spongy bone give a very clear crystalline pattern as shown diagrammatically in figure 2 (*e.f.g.*) and the spacings associated with the pattern are given in table 1.

While this work was in progress a very complete article on x-ray analysis of bones and teeth (6) appeared in which the spacings present are calculated from powder photographs. The monochromatic pinhole method is essentially qualitative so that the spacings given in table 1 are much less accurate than those given by Roseberry and his co-workers (6) but a comparison of the more intense lines shows very good agreement (see table 2).

Although the monochromatic pinhole method does not give very exact measurements of spacings it gives interesting information about the alignment of crystals in bone in its natural state. It is evident from figure 2

and table 1 that the apatite crystals present in compact bone are oriented giving a fiber structure (spacing 3.4 and 2.25) especially in the forearm bone of the rat. With the x-rays perpendicular to the fiber direction (longitudinal section of bone) these spacings appeared as arcs and, with the x-rays parallel to the fiber direction, these spacings were faint and appeared as rings. The strong spacing 2.8 A. U. showed no evidence of fibering and as it is identical in spacing with the collagen in tendons and cartilage it has been assumed that it is due, in part at least, to the presence of unoriented crystals of collagen. The presence of a strong apatite spacing at 2.72 A. U. makes it probable that the strong continuous ring giving the spacing 2.8 A. U. with all bone sections may be due partly to apatite but the identity with the collagen spacing, the lack of fibering in this ring, and the fact that collagen has been found to exist in crystalline form under certain conditions,

TABLE 2

Spacing in A. U. from x-ray spectrograms of bone, teeth, apatite

ROSEBERRY, HASTINGS, AND MORSE						CLARK			
Fluor-apatite	Chloro-apatite	Dahlite (carbon-ate apatite)	Enamel	Dentine	Bone	Bone long section	Bone cross section	Dentine	Enamel
								5.2	5.2
	3.35	3.35	3.35	3.35	3.35	3.4	?	3.5	3.5
2.72	2.72	2.72	2.72	2.72	2.72	2.8	2.8	2.8	2.7
2.60	2.60	2.60	2.60						
2.25	2.26	2.24	2.25	2.25	2.25	2.25	2.25	2.35	
1.71	1.71	1.71	1.71	1.71	1.71	1.7	1.75	1.7	
1.64	1.64	1.62	1.64			1.6			
1.44	1.44	1.44	1.44			1.4	1.4	1.4	

suggests the possibility that in bones, owing to dehydration, change in pH, or some other physical cause, the collagen present in cartilage in amorphous condition goes into a crystalline form which has been given the name of ossein. It is therefore tentatively assumed that unoriented organic crystals (collagen or ossein) and oriented inorganic crystals (apatite) are present in bone.

Teeth. Thin longitudinal sections were cut from swine's teeth so that diagrams could be obtained from dentine and enamel separately. The patterns obtained are shown in figure 2 (*h*, *i*) and the spacings are given in table 3. The spacings are similar to those found in bone, the pattern of dentine being similar to a cross section, and that of enamel to a longitudinal section of a compact bone, the enamel showing marked fibering in the direction of the length of the prisms. In addition there is a new spacing 5.2 A. U. which is clearly due to apatite as it was also obtained from phos-

phorite. This spacing is more pronounced in enamel than in dentine and the ring which has been assumed to be at least partly due to collagen, spacing 2.8 A. U., which is still very strong in the dentine pattern, becomes faint in the enamel pattern and is shifted to 2.7. Since this value coincides with an apatite spacing and lies very close to the second order of the 5.2 spacing (probably the 2.6 spacing given in table 2), and as the ring is slightly asymmetrical, it is probable that the b spacing in enamel is due altogether to apatite and that in enamel there are no organic but only inorganic crystals present. It seems therefore that dentine in teeth is composed partly of inorganic crystals of apatite and partly of organic crystals of collagen with no definite orientation in either. Enamel seems to contain no crystalline collagen but to have inorganic crystals of apatite oriented in fiber fashion with respect to the enamel prisms.

TABLE 3

(Teeth)

	DENTINE (SWINE)	ENAMEL (SWINE)	PHOSPHORITE	SPACING A.U.
X	Ring (faint)		Ring	5.2
		Ellipse (stronger than in dentine)		5.2-5.4
a	Faint arcs (doubtful)	Clear arcs		3.5
b	Strong continuous ring			2.8
		Continuous ring (not as strong as dentine)		2.7
			Ring (2nd order X)	2.6
c	Faint ring	Very faint ring		2.35
d	Faint diffuse ring	Very faint ring		1.7
f	Faint ring (probably 2nd order b)	Very faint ring		1.4

Decalcified bone and tendons treated with trypsin. Tendons treated with trypsin (18–42 hours) to remove albumoid and mucoid material, and exposed moist, showed the same zone (spacing 3.0–3.1 A. U.) as in fresh material, except that a spot on the edge of the zone in the direction of the length of the tendon showed the appearance of microcrystalline collagen in tendon treated with trypsin.

Bones, decalcified in 5 per cent nitric to remove calcium salts, showed only amorphous material with a spacing of 3.0 A. U. This observation has been made before and has been taken to mean that all the crystals present in normal bone are due to its inorganic constituents. It is just as tenable however to assume that prolonged treatment with acid not only removes the inorganic crystals, but also decrystallizes the crystalline collagen, leaving it in an amorphous condition. A piece of decalcified bone, thoroughly

rinsed in water and pressed in a clamp while drying, showed a pattern similar to dried tendon with two arcs in the direction of the length of the bone with the collagen spacing 2.8 A. U. This shows that after decalcification crystalline collagen may still be produced in bone if dried under tension, and that the spacing 2.8 A. U. present in normal bone is probably due, in part at least, to organic crystals of collagen.

Densitometer curves. As the x-ray photographs do not reproduce very well densitometer curves were made from the films with a Moll recording microphotometer. In this instrument a beam of light passes through the film before falling on a thermopile, so that as the film passes in front of the light the current generated in the thermopile varies with the density of the image in the film. The densitometer curve is a record of this current given by the recording galvanometer and measures the density of the photographic image on the films. Unfortunately the length of exposure necessary for obtaining the x-ray diagram made a general diffuse darkening near the centre spot which almost masks the zone in the tendon and cartilage diagrams.

Figure 3 represents the densitometer curves in two directions through the diffraction pattern of a fresh tendon stretched in the direction of its length such as shown in figure 2 *b*. The top curve 109 B is at right angles to the stretching direction giving an indication of the amorphous zone in the position of the arrows. The densitometer curves for fresh unstretched tendon, figure 2 *a*, are similar to this in both directions. The bottom curve in figure 3 (109 A) is in the direction of length of the stretched tendon and the humps indicated by the arrows show the spots at the edge of the amorphous zone which indicate the presence of microcrystals.

Figure 4 represents curves in two directions through the diffraction pattern of a stretched dried tendon such as shown in figure 2 *c*. At right angles to the stretching direction the amorphous zone has disappeared (upper curve 106 B). In the stretching direction the crystal arcs still persist as shown by the humps under the arrows (bottom curve 106 A).

The bottom curve in figure 5, taken from costal cartilage, shows only the amorphous zone (arrows 2) characteristic of amorphous collagen. The upper curve in figure 5 was taken from dentine. The center arrows show the apatite ring (spacing 5.2 A. U.). The arrows marked 2 show the strong ring attributed to crystalline collagen. The faint ring with spacing 2.36 does not appear but the ring that is given the notation d in table 3 is indicated by arrows.

In figure 6 the densitometer curves of the diffraction pattern from a longitudinal section of compact bone (fig. 2 *e*) are given in two directions. The bottom curve (156 A) is in the direction of the length of the bone. Arrows 1 indicate the arcs (spacing 3.35). Arrows 2 give the crystal collagen ring. Arrows 4 indicate the ellipse d. The upper curve (156 B) gives the curve

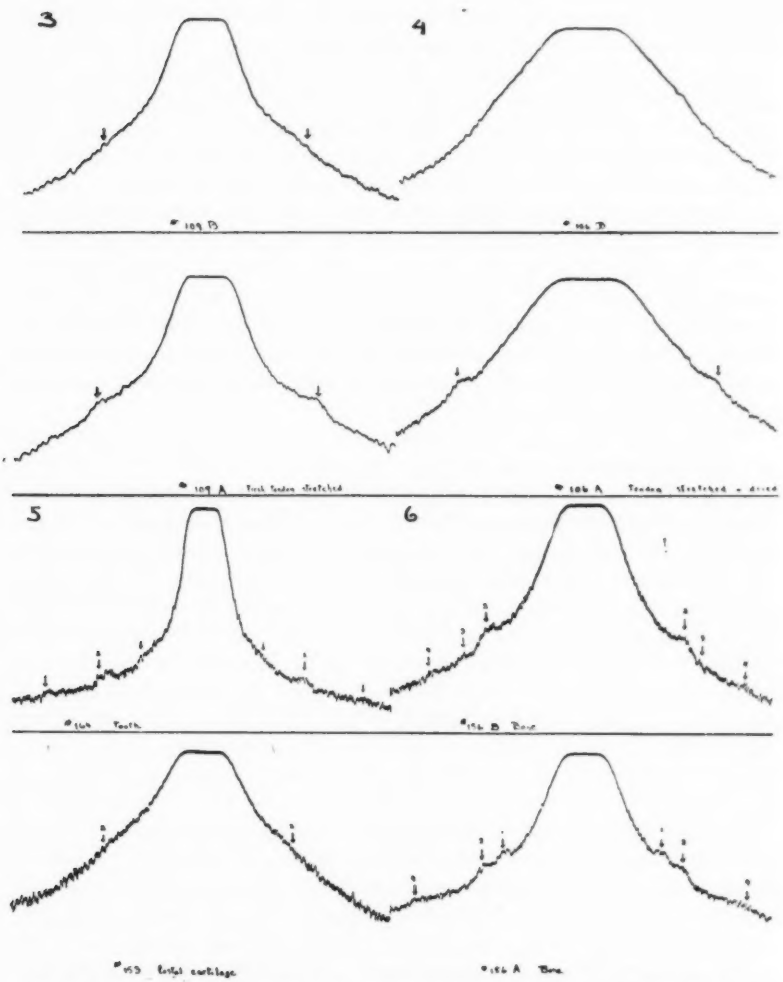

Fig. 3. Densitometer curves for stretched living tendon (109 A) in stretching direction (109 B) at right angles to stretching.

Fig. 4. Densitometer curves for stretched dried tendon, (106 A) stretching direction, (106 B) at right angles to stretching.

Fig. 5. Densitometer curves for (153) costal cartilage, (164) dentine.

Fig. 6. Forearm bone of rat (156A) length of bone, (156 B) at right angles to length.

from the same film at right angles. Spacing a is absent but spacing b (crystal collagen ring) is given by arrows 2. The faint arcs, *c*, appear, marked by arrows 3 and arrows 4, give the ellipse *e*.

Discussion. The conclusion drawn from the above observations is that collagen exists in normal unstretched tendons and in cartilage in amorphous condition but that the colloidal micellae may become crystalline.

It is a debatable point whether the appearance of spots on a zone, as in stretched tendon, indicates the appearance of crystals or the orderly arrangement, under external forces, of what some have termed crystalline molecules (i.e., large molecules with a regular internal structure capable of diffracting x-rays like a microcrystal). The German investigators have usually assumed that the appearance of arcs and rings in the x-ray diagrams of organic material indicates the appearance of microcrystals. It is therefore possible to assume that the colloidal micellae of collagen can be converted into microcrystals by external force (such as stretching) by drying, and probably by other means.

The development of crystals in stretched tendons increases their tensile strength and marks the limit of their elasticity.

In non-ossifying cartilage collagen is present in amorphous form but as the unoriented crystals present in bone have the same spacing as collagen it is suggested that collagen exists in bones in crystalline condition and that this crystalline collagen is presumably identical with ossein. This change of collagen from amorphous to crystalline form in bones might be brought about by changes in state of hydration of the colloidal micellae or by changes in acidity. Owing to the fact that the inorganic crystals present (apatite) have a spacing nearly identical with the 2.8 spacing attributed to crystalline collagen, the presence of these organic crystals is not proved but only suggested by this work. If collagen changes to crystalline form in bones this change of state might be an important factor in the process of bone formation.

Cartilage has been found to exhibit a marked adsorption for calcium salts due to its colloidal condition so that as long as collagen is present in colloidal form there would be an accumulation of calcium salts from the blood, up to, or near the limit of saturation. If now, from a change in acidity, or any other cause, the collagen were to become crystalline it could no longer hold this large concentration of calcium salts in solution and they would precipitate. Robison and his co-workers (9) have shown that there are two mechanisms involved in normal calcification. One is the phosphatase mechanism by which an enzyme present in ossifying bone hydrolyses phosphoric ester, thereby increasing the inorganic phosphorus and producing a condition of supersaturation. The second, which he calls the "inorganic mechanism," is a factor of unknown nature which favors the deposition of calcium phosphate from supersaturated solutions. This

second factor might very well be the presence of organic crystals of collagen or ossein. This second mechanism was found to be destroyed by soaking bone slices 24 hours in alcohol, or chloroform, but slices of bone treated in this way gave normal diffraction patterns which tended to disprove rather than prove the above hypothesis. But crystalline collagen, if present in bone, would act as an inorganic mechanism favoring the precipitation of salts and the change in adsorptive power of collagen for calcium salts, in passing from the colloidal to the crystalline condition, may be looked upon as one of the physico-chemical factors involved in the process of bone formation.

CONCLUSION

X-ray diagrams of bones and teeth, by the monochromatic pinhole method, show that inorganic crystals of apatite are present in bone oriented so as to give fiber structure in longitudinal section. There are also unoriented crystals present which are attributed to organic crystals of collagen, or ossein.

Dentine was found to contain unoriented inorganic crystals of apatite and also, probably, unoriented crystals of collagen.

Tooth enamel was found to contain only inorganic crystals of apatite, oriented with respect to the prisms so as to give a fiber pattern.

The possible importance of crystalline collagen in bones, as one of the physico-chemical factors involved in the process of bone formation, is discussed.

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THE TACHYCARDIA, TIME FACTOR, SURVIVAL PERIOD AND SEAT OF ACTION OF THYROXINE IN THE PERFUSED HEARTS OF THYROXINIZED RABBITS

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The rather frequent occurrence of cardiac arrhythmia and of congestive heart failure due to hyperthyroidism has served to stimulate great interest of late in the problem of the fundamental nature of the impairment of cardiac function.

The tachycardia. The tachycardia in hyperthyroidism has always attracted attention. It is one of the cardinal signs of the condition. Its intensity bears a rough but not exact relationship to the basal metabolic rate. The heart rate is relatively invariable, i.e., it remains rapid with the patient at complete rest. The cause of the tachycardia had usually been thought to be a stimulation of the sympathetic nerves, an increased production of adrenalin or toxic influences on the myocardium.

Several months after the present study had been begun and just at the time of its completion, J. K. Lewis and McEachern (1931) published the results of experiments with rabbits' hearts which showed that the isolated hearts of thyroxinized animals persist beating at an accelerated rate. More recently Priestley, Markowitz and Mann (1931) have published a report of work along similar lines. They found also that the hearts of thyroxinized rabbits when perfused beat faster than normals and were apt more often to be arrhythmic.

The present study was undertaken in the summer of 1930 and carried on through the fall and winter. It was first desired to determine whether the hearts of thyroxinized animals beat faster because of the effect of thyroxine upon the accelerator nerves or because of some other extra-cardiac factor or whether it may be that the accumulation of thyroxine in the heart itself is responsible. A series of normal rabbits was first studied. The animals were rapidly killed by bleeding. The heart was removed and washed out through the aorta with Ringer-Locke's solution. It was then perfused through the coronaries by means of a cannula in the aorta according to the well known method of Locke and Rosenheim (1907) at a gravity fall of about 65 cm., and at a temperature range between 37° and 38°C. The heart rate was counted by a stop watch every 10 minutes for one hour

and a half. After the perfusion had begun it usually required about 15 minutes before the heart rate became regular. Occasionally a period of irregularity would develop during the experiment. The hearts of thyroxinized rabbits often required more time to become regular or developed more frequent attacks of arrhythmia. The rates of different hearts varied from 106 to 174 per minute with an average rate of 23 hearts of 140

TABLE 1

(A) AVERAGE RATES PER MINUTE OF PERFUSED HEARTS OF NORMAL AND THYROXINIZED RABBITS, COUNTED AT 10 MINUTE INTERVALS FOR 1½ HOURS				(B) EFFECT OF TIME ON RATE OF HEART BEAT AFTER THYROXINATION WITH 0.5 MGM. PER KILO	
Number	Normal	Thyroxinized	Dose	Day	Average rate
1	142	175	1.0 mgm.	1	142
2	131	195	1.0 mgm.	2	152
3	144	194	1.0 mgm.	3	171
4	106	202	1.0 mgm.	4	169
5	139	175	1.0 mgm.	5	192
6	126	193	1.0 mgm.	6	195
7	149	194	1.0 mgm.	7	187
8	160	203	1.0 mgm.	8	225
9	144	192	1.0 mgm.	9	192
10	174	202	1.0 mgm.	10	174
11	148	150	0.5 mgm. per K.	11	179
12	131	202	0.5 mgm. per K.	12	*
13	145	211	0.5 mgm. per K.	13	205
14	106	155	0.5 mgm. per K.	14	171
15	138	203	0.5 mgm. per K.	15	154
16	126	227	0.5 mgm. per K.	16	131
17	116	204	1.0 mgm. per K.	17	152
18	114	214	1.0 mgm. per K.		
19	138	207	1.0 mgm. per K.		
20	170	207	1.0 mgm. per K.		
21	148	211	1.0 mgm. per K.		
22	158	128	1.0 mgm. per K.		
23	159	191	1.5 mgm. per K.		
Average...	140	193	Per cent increase 38		

* Rabbit died.

(table 1, A). Usually the rate of the individual heart was fairly constant, but in a few there was considerable variation, usually within the limits given.

Another series of rabbits was then given thyroxine intravenously, each 1.0 mgm., and 5 days later the hearts were perfused under the same conditions as the normals. Squibb's natural thyroxine dissolved in slightly alkalinized normal saline solution was used. There was a striking in-

crease in rate over the normal hearts, the rate ranging from 128 to 227 per minute, with an average rate of 23 hearts of 193, or an increase of 38 per cent above the average of the normal hearts (table 1, A). Other rabbits were injected with thyroxine intravenously in doses of 0.5, 1.0, 1.5 mgm. per kilo of body weight. The same degree of tachycardia was present as with those injected with 1.0 mgm.

The time factor. In order to determine the duration of this effect and the time factor on its intensity a series of 17 rabbits was given 0.5 mgm. per kgm. thyroxine intravenously at the same time, and one rabbit heart perfused daily thereafter. It had previously been learned that there is a latent period of about two days before a definite increase in rate of heart beat occurs. The increase in rate sets in rather rapidly after the second day and persists for about 12 days, after which it rapidly declines to the normal range (table 1, B).

The survival period. It was desired next to determine whether the perfused hearts of thyroxinized rabbits would beat as long as those of non-thyroxinized rabbits. A great difficulty in this type of experiment is the invariable development of extensive edema after a few hours of perfusion. However, a comparative study may be of some value. It is important to establish the end point of such an experiment. In this series the end was considered to have been reached when the heart beat was extremely weak, or very weak and very slow, or practically obscured by edema. Ort and Markowitz (1931) have discussed some of the factors responsible for such edema. The survival period of 7 normal perfused hearts ranged from eight hours and five minutes to twelve hours and thirty minutes. Irregularity was seldom seen after the initial half-hour of the perfusion. The survival period of the hearts of thyroxinized rabbits was not appreciably different. The results of the total series of 12 hearts irrespective of the dosage showed a survival period of from five hours and fifty-five minutes to fourteen hours and thirty minutes. This compares favorably with the normals and indicates that with the dosages used (0.5, 1.0 and 1.5 mgm. per kgm.) the heart of the acutely thyroxinized rabbit will beat as long when perfused as the heart of the non-thyroxinized rabbit. The result with rabbits kept hyperthyroid for long periods of time might be different. The doses of thyroxine used are sufficient to produce a high grade of hyperthyroidism; with much larger doses the result might also be different. In these experiments it was found that great care in thoroughly cleaning the perfusion apparatus is essential. The survival period is greatly shortened by even slightly dirty glassware. This has also a definite but slight effect upon the heart rate.

The seat of action. The points to be determined in this phase of the problem were whether the tachycardia could be ascribed to the effect of thyroxine upon the sino-auricular node or upon the muscle mass, and if on

the latter whether the effect is exerted upon the ventricles as well as the auricles.

Rabbits apparently normal and rabbits which had received intravenously 0.5 mgm. per kgm. of Squibb's natural thyroxine (dissolved in slightly alkalinized normal saline solution) five days before the experiment were used. The heart was removed and perfused as in the preceding experiments. It was allowed to beat undisturbed until the rhythm became regular and the rate fairly well established. Then that portion of the auricle containing the sino-auricular node, together with a portion of the roof of the left auricle, was excised. Following this the heart rate was counted at intervals for about an hour, and then the upper portion of the interventricular septum was clamped tightly for a brief period with the tip of a small hemostatic forceps. This was admitted through the orifice

TABLE 2

Average ventricular rate of perfused hearts of normal rabbits and of rabbits five days after intravenous injection of 0.5 mgm. thyroxine per kgm., before and after excision of the sino-auricular node and after crushing the bundle of His

NUMBER	NORMAL			THYROXINIZED		
	Initial rate	After excision of S.A. node	After crushing of His bundle	Initia rate	After excision of S.A. node	After crushing of His bundle
1	131	87	61	152	138	114
2	122	89	55	237	207	138
3	172	141	111	264	201	132
4	149	89	31	197	112	125
5	153	99	72	221	138	44
6	148	109	49	197	190	79
Average..	146	102	63	211	164	105

produced by the previous partial excision of the auricles so that the auriculoventricular bundle was necessarily completely severed by the crushing. The rate of beating of the ventricles was carefully counted at intervals for another hour, and an attempt was made to count auricular beats simultaneously. The latter did not prove easy to do.

Some very interesting facts were brought to light. With the normal hearts (table 2) there was often a marked slowing of the heart rate after excision of the sino-auricular node, say from 140 to 100, but the rhythm was for the most part quite regular. After crushing of the bundle of His the ventricles usually slowed down to a rate between 40 and 80 with regular rhythm, the auricles beating independently at a faster rate. With the hearts of thyroxinized rabbits (table 2) there was usually a slowing of the heart rate after excision of the sino-auricular node, but even so the rate was

usually more rapid than the heart rate of the normal undisturbed perfused heart, and the reduction in rate was about proportionate to that of the normal heart after excision of the node. When the bundle of His was clamped there was a diminution in rate of the ventricles, and the decrease was about as great proportionately as in the normal, but usually the ventricular rate was nearly that of the normal undisturbed perfused heart. In two instances, however, the ventricular rates were quite slow and within the same limits as the normal hearts after crushing of the bundle. The auricular rate was difficult to be certain of. Sometimes the auricles seemed to beat at about the same rate as the ventricles, sometimes even synchronously, and once at a rate much slower than the ventricles, but usually they were faster.

Discussion. This study has led us independently to arrive at the same conclusion as other workers in regard to the tachycardia, viz., that the accelerating effect of thyroxine on the heart rate persists in the isolated perfused heart. This fact indicates that, although the increase in heart rate in hyperthyroidism may be important as a factor in the increased circulatory rate, it is not primarily a compensatory condition but is probably due to the direct effect of thyroxine upon the heart. In other words, the tachycardia is a manifestation of the effect of thyroxine upon a pulsatile organ, and is probably due to the increased irritability which accompanies the elevated metabolism of the organ.

The finding that the accelerating influence of a single dose of thyroxine on the rate of the isolated heart can be manifested as long as seventeen days may be compared with the prolonged effect of a single dose of thyroxine on the myxedematous patient. Boothby (1929) states that 14 mgm. of thyroxine given intravenously will raise the basal heat production of a myxedematous subject for six to ten weeks.

These experiments show that in the case of the acutely thyroxinized animal, with the dosages employed, the heart beats as long and probably more vigorously than the normal heart. Other experiments are now being made to determine the effect of prolonged thyroxinization on the life of the isolated heart. It is logical to assume that a heart which has been beating faster and more vigorously than normal for a long period of time should more quickly go into a state of fatigue when perfused.

While these experiments indicate that thyroxine acts upon all parts of the heart, they do not settle the question as to whether the action is directly upon the muscle fibers or upon the nerve endings in the myocardium. The work of Cecile Markowitz on tissue cultures of hearts of chick embryos, indicates that thyroxine acts upon the muscle cell directly. This work is being done on cultures explanted before the development of intrinsic ganglia.

SUMMARY

1. Perfused hearts of acutely thyroxinized rabbits beat at a much faster rate than hearts of non-thyroxinized rabbits, as has also been demonstrated by J. K. Lewis and McEachern (1931) and by Priestley, Markowitz and Mann (1931).

2. There is a latent period of about 2 days following intravenous injection of thyroxine before this acceleration is noted.

3. As shown by serial perfusion experiments, the heart continues to beat at a very rapid rate for about 12 days and then returns to the normal rate in about 17 days after the injection. Within certain limits dosage does not seem to make much difference in regard to the degree of tachycardia. This probably means that the smallest dose used was maximal.

4. The survival period of rabbits' hearts sufficiently thyroxinized to make them acutely strongly hyperthyroid is not significantly different from the survival period of non-thyroxinized rabbits' hearts.

5. It has been noted in the experiments that the hearts of thyroxinized animals were more subject to attacks of arrhythmia and seemed to beat more vigorously than normal.

6. After excision of the sino-auricular node of the perfused heart of the acutely thyroxinized rabbit, the heart rate is still greatly increased. When auriculoventricular dissociation is produced by crushing the bundle of His the ventricles usually beat at an accelerated rate. These results mean that the auricles and ventricles independently beat faster than normally. Thyroxine seems to exert its effect, therefore, upon all parts of the heart.

7. This study does not indicate whether the action of thyroxine is upon the muscle fibers or upon the nerve endings in the myocardium, but other studies now being made (Cecile Markowitz) give reason to believe that it is directly upon the muscle cells.

Acknowledgment is given of the very valuable technical assistance of Stephen E. Kramer, Jr. B.S. (Med.).

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AN EXPERIMENTALLY PRODUCED PREMATURE SYSTOLIC ARHYTHMIA (PULSUS BIGEMINUS) IN RABBITS

IV. Effective Areas in the Brain

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The purpose of this investigation is: *a*, to determine if faradic or osmotic-chemical stimulation of certain areas of the forebrain and midbrain known to yield vascular and respiratory changes, will also produce a premature systolic arhythmia; *b*, to determine if a premature systolic arhythmia can be evoked from insufflations into the nostrils after the brain had been transected through the mesencephalon.

Procedure. All faradic stimulations were made with a 2 to 3 volt battery and a Harvard inductorium. Contacts were established by a bipolar electrode, the points of which were 1 to 2 mm. apart. Every precaution was taken to prevent spread of current to other areas. The osmotic-chemical excitations were affected by injecting 0.05 cc. of 7 per cent sodium citrate solution into the area to be stimulated after the method of Maxwell. Control records were taken during and following the application of the citrate solution to the dura, the insertion of the needle, the injection of normal saline into the area to be stimulated and the injection of the citrate solution into adjacent areas. The regions concerned were well supplied with blood, both vertebrals and one carotid were always intact unless otherwise stated. The animals were given a two-thirds of an anesthesia dose of barbital sodium intravenously and all operations were performed under light ether. Respiratory tracings were taken from the trachea or thorax and blood pressure was recorded from the carotid. All brains were carefully fixed and preserved.

Motor area of the cerebrum and internal capsule. It is well known from the experiments of a dozen investigators that faradization of a median cephalic area of the cerebrum produces pronounced vascular and respiratory changes in the monkey, dog, cat and rabbit, but the changes reported are conflicting. This area (fig. 1A, *D*) was stimulated many times in 11 rabbits with varying intensities of current. Weak and strong excitations (secondary 10 to 6 cm.) were nearly always followed by a drop in arterial pressure. The pulse showed little change from weak stimulation, but was strengthened and slowed considerably by strong excitation. Respiratory

movements were invariably inhibited or arrested. Only very strong faradizations (5 and 4) produced a tonic spasm and clonic convulsions. No premature sytoles were induced from faradization of the motor area. In 2 animals faradic stimulation of the internal capsule elicited the same response as noted above for the motor area.

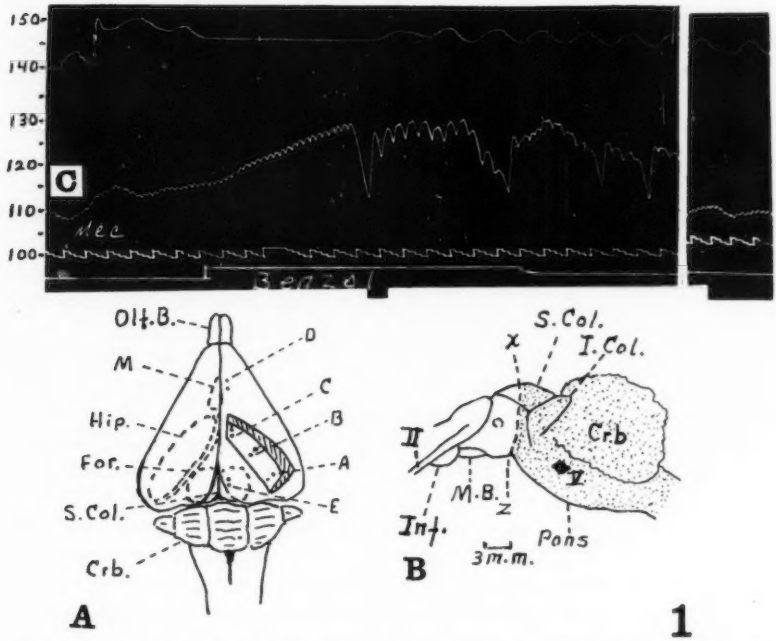

Fig. 1 A. Rabbit brain from above, hippocampus exposed on right side. On left side the position of the motor cortex, hippocampus and superior colliculus indicated by dotted lines. *A* to *E* show areas stimulated. Abbreviations: *Olf. B.*, olfactory bulb: *M.*, motor cortex; *Hip.*, hippocampus; *For.*, fornix; *S. Col.*, superior colliculus; *Crb.*, cerebellum.

1 B. Shows plane of transection in rabbit 810. The light area in front of the line *x-z* was removed. Abbreviations: *S. Col.*, superior colliculus; *I. Col.*, inferior colliculus; *Crb.*, cerebellum; *Inf.*, infundibulum; *M. B.*, mammillary bodies; *II*, optic chiasma; *V*, trigeminal root.

1 C. Thoracic respiratory and carotid tracings at the time of benzol insufflation from rabbit 810 after the transection *x-z* had been made. Omitted portion represents a 51 second interval of arhythmia, time in seconds.

Osmotic-chemical excitations of the white matter of the motor area of the cerebrum (fig. 1A, *D*) in 4 animals was followed in a few seconds by 17 to 40 mm. rises in arterial pressure, a slowed and strengthened pulse.

but no premature systoles. At first there was a tonic spasm of about 30 seconds duration, during which respiration was inhibited and arrested. This was followed by a much longer clonic phase in which respiration was deepened, accelerated and irregular. The spasm came on simultaneously with the changes in respiration and circulation. The use of weaker solutions of sodium citrate or injecting the standard solution into areas close to the motor cortex elicited a weaker and delayed response in which the interval of tonic spasm and arrested respiration was sometimes omitted. Normal saline injected into the motor area and 7 per cent sodium citrate injected into the cerebrum above the hippocampus, behind and lateral to the motor area seldom produced any vascular or respiratory changes.

Superior colliculi. It has been demonstrated by Danilewsky, François-Frank, Christiani, Martin and Booker, Knoll, Sachs, Brown, Schrottenbach, Miss Coombs, Allen and others that faradization of the superior colliculus or the tecto bulbar tract causes marked changes in respiration and circulation. Weak faradization (9 and 8 cm.) of the superior colliculus, *E*, (fig. 1A) directly or through or between the occipital lobes disclosed moderate rises in blood pressure and pulse which are comparable to those obtained from benzol insufflation, but no premature systoles. Respiration was altered considerably and the general spasm and convulsions well described by Polimanti usually continued for some time.

Stronger stimulation (7 or 6) generally yielded a rise in arterial pressure of 37 to 40 mm. and a pronounced slowing and strengthening of the pulse. In one record two short intervals of premature systolic arhythmia followed a 40 mm. rise in blood pressure and a record from another animal revealed a few premature systoles following a 37 mm. rise. However, the great majority of the graphs taken from a number of animals showed no arhythmia. The same and even stronger response was obtained from an osmotic-chemical stimulation of the colliculus.

To determine if the enormous rise in blood pressure obtained in some of the above records from strong stimulation was responsible in any way for the arhythmia, the superior colliculus was stimulated with a much stronger tetanus current while blood pressure was maintained at a constant level by an equalizer attached to the abdominal aorta. In ten animals in which this procedure was used there was no evidence of premature systoles when blood pressure was kept below a 5 mm. rise.

Olfactory areas. According to Ducceschi, faradization of the olfactory bulbs was followed by inhibition of respiration, while stimulation of the pyriform lobe produced excitation. Sachs observed that faradic stimulation of the hippocampus and fornix elicited no vascular or respiratory response, but excitation of the habenula bodies usually resulted in a rise in blood pressure and inhibition of respiration. In the first paper of this series the writer reported a moderate rise in blood pressure and an occasional bigeminal pulse from peripheral stimulation of the olfactory nerve.

The olfactory bulbs of 10 rabbits were faradized many times with different intensities of current without any sign of premature systoles. Weak excitations (9 and 8) evoked no vascular or respiratory changes; stronger (7 and 6) generally resulted in identical responses to those obtained from the motor cortex; while still stronger (5 and 4) may be followed by the cortical motor type of response or a response that is very similar to the one obtained from peripheral stimulation of the trigeminal nerve. Further experiments in which the olfactory bulbs were transected and left touching the cerebrum and others in which the bulbs were completely isolated from the cerebrum demonstrated that the effects from the stronger excitations were in all probability due to a spread of the current to the motor area or to the trigeminus.

Excitation of the central area of the olfactory bulbs in 5 rabbits by the osmotic-chemical method ordinarily evoked very pronounced vascular and respiratory responses, but no arhythmia or sneeze. The general response was very similar to the one obtained from like stimulation of the motor area of the cerebrum. Control tests in which the sodium citrate solution was applied to the meninges and injected into a bulb after the bulbs had been severed from the cerebrum were not followed by any response.

The dorsal or proximal portion of the hippocampus was exposed (fig. 1A, right side) in 11 rabbits by removal of a narrow strip of the cerebral cortex. This dissection should not interfere with any of its fiber connections. Weak faradizations (9 and 8) at the points *A*, *B* and *C*, figure 1 A and elsewhere did not result in any appreciable changes in arterial pressure, pulse or respiration. Stronger stimulations usually induced the motor cortex type of response and further experiments indicated this response to be due to a spread of the current to the motor area. There was no evidence of premature systoles in any of the records.

Osmotic-chemical excitations of the deeper portion of the hippocampus in 5 rabbits were followed by the same general reactions as were obtained from like stimulation of the olfactory bulbs and the motor area of the cerebrum. A premature systolic arhythmia occurred in one record in which there was a sufficient rise in blood pressure (62 mm.) to account for the arhythmia. There is obviously some possibility of stimulating other areas during injection of the hippocampus, but injecting the adjacent cerebral cortex and flooding the surface of the hippocampus with the sodium citrate solution has not produced any vascular changes.

Both habenula bodies were stimulated (secondary at 7) in 2 rabbits. The result was a 10 to 12 mm. rise in blood pressure, little or no pulse change, respiratory movements strengthened and rate slowed or unchanged, but no premature systoles.

Hypothalamus. Recent investigations have shown this area to be most

important for regulating visceral functions. Within a year Beattie, Brow and Long have reported an extrasystolic arhythmia in cats under chloroform anesthesia from faradization of the posterior part of the lateral wall of the third ventricle. In addition they traced retrograde degenerated fibers from the area of stimulation to the pyriform lobe and efferent descending fibers to the reticular formation and to the lateral gray columns of the spinal cord.

Similar areas of the hypothalamus of 4 rabbits were faradized. Three of these animals yielded premature systolic arhythmias, which in some instances continued for several minutes and one gave a rise in blood pressure but no arhythmia. Ordinarily the arhythmias were preceded by a small or moderate rise in blood pressure.

Effect of transections of the mesencephalon on the bigeminal arhythmia from insufflations. Brow, Long and Beattie found that premature systoles following chloroform anesthesia were abolished in the cat if the diencephalon was transected in a plane extending from the cephalic border of the superior colliculi to the caudal border of the mammillary bodies. In another paper they report that a ventro-dorsal stab wound, which extends 2 mm. to either side of the midline through or behind the mammillary bodies in the direction of the cephalic border of the superior colliculi to the depth of the aqueduct, was sufficient to abolish ectopic beats that followed chloroform anesthesia administered through a tracheal cannula.

The following procedure was adopted in this experiment. Cannulae were inserted into the trachea, pharynx, right carotid and the left carotid was ligated. A transverse cut was made through the hemispheres and mesencephalon with a blunt pointed scalpel and all of the brain in front of the cut was removed with a spatula. Care was exercised not to injure the trigeminal roots and ganglia. Initial experiments disclosed the importance of obtaining insufflation records as soon as possible after transecting the mesencephalon to avoid the effects of hemorrhage, anemia, etc., which may prevent a bigeminal arhythmia from insufflations. The exact plane of transection was determined after the brain had been thoroughly fixed.

Premature systolic arhythmias have followed benzol insufflation from 7 rabbits in which the mesencephalon had been transected. In 4 animals the plane of transection passed close to the center of the superior colliculi through the mesencephalon to the pons or within a millimeter of it. In 3 animals the plane of transection passed through the cephalic border of the superior colliculi to a point 1 mm. caudad of the mammillary bodies.

In the animal selected for descriptive purposes the plane of transection is as (fig. 1B, *x-z*) throughout, extending to the level of the pons at the midline. A premature systolic arhythmia (fig. 1C) obtained after the transection (*x-z*) discloses the usual moderate rise in blood pressure, pulse and respiratory changes which accompany arhythmias from insufflations.

The omitted portion of the tracing represents a 51 second interval of continuous premature systoles. Upon becoming normal the pulse remained so for 5 minutes, when a second insufflation of benzol elicited a second premature systolic arhythmia which lasted for more than a minute. In several instances transecting the mesencephalon seemed favorable for a long continuation of the "insufflation arhythmia" or for the appearance of several short intervals of this arhythmia before the normal is regained.

Discussion. It seemed obvious to the writer that the premature systoles occasionally obtained from strong faradization of the superior colliculus and from occasional osmotic-chemical stimulations were to be attributed to the accompanying enormous rise in blood pressure, but as was pointed out by Mr. Goodman, there is a possibility of a difference in the arhythmia threshold from trigeminal and superior colliculus stimulation which might account for the latter coming on occasionally with the very strong excitations. When, however, the rise in blood pressure was eliminated as a factor for production of the arhythmia by the introduction of an equalizer, premature systoles never followed very strong stimulations of the superior colliculi. The statement made in the second paper of this series that the bigeminal arhythmia obtained from insufflations is not contingent on the moderate rise in blood pressure which precedes it, is not to be construed to mean that a sufficiently high rise in blood pressure will not occasionally elicit an ectopic arhythmia in rabbits; for it has been shown by Heidenhein, Dogiel and others that complete occlusion of the abdominal aorta sometimes produces a premature systolic arhythmia.

Beattie, Brow and Long's results from faradization of the hypothalamus were confirmed in rabbits. That the hypothalamus is not the only area of the brain stem in rabbits from which this arhythmia can be induced is shown by the previous experiment where the arhythmia from insufflations was readily obtained after the mesencephalon had been transected. Indirectly this experiment contributes further data to substantiate the importance of the entire formatio reticularis of the brain stem and various nuclei derived from it for altering circulation, respiration and for the production of convulsions.

The following observations while having no particular bearing on this problem should be of general interest: 1. Osmotic-chemical excitations of the motor area of the cerebrum were usually followed by a pronounced rise in arterial pressure, a slowed and strengthened pulse, a short tonic spasm in which respiration was inhibited and arrested and a longer period of clonic convulsions in which respiration was deepened, accelerated and somewhat irregular. Very strong faradizations of the same area resulted in the same response, except that there was a drop in arterial pressure; while weak or moderate faradization of the motor cortex nearly always produced a drop in blood pressure, little or no pulse changes, an inhibition of respiratory move-

ments, but no spasms or convulsions. 2. In the main Sachs' observations are confirmed that faradization of the habenula bodies caused a small rise in blood pressure and inhibition of respiration (my tracings showed a deepening and a slowed or unchanged rate); while similar excitations of the hippocampus evoked no response. 3. Weak faradization of the olfactory bulbs produced no vascular or respiratory changes. On the other hand, osmotic-chemical irritation of the deeper parts of the olfactory bulbs and hippocampus generally induced the same response as recorded above for like stimulation of the motor area.

SUMMARY AND CONCLUSIONS

Weak faradization of the motor area of the cerebrum, superior colliculi, olfactory bulbs, hippocampus and habenula bodies (2 animals only) did not produce premature systoles.

Osmotic-chemical excitations of the deeper portions of the olfactory bulbs, motor area and hippocampus seldom resulted in premature systoles.

For the reasons stated in the discussion, the few instances in which a premature systolic arhythmia followed strong faradization of the superior colliculus or some of the osmotic-chemical stimulations, can in all probability be attributed to the accompanying enormous rise in blood pressure.

A premature systolic arhythmia was elicited in three rabbits from faradization of the hypothalamus.

The premature systolic arhythmias obtained from benzol insufflation after the mesencephalon had been transected, demonstrates a trigeminal connection to an efferent center below the diencephalon for inducing this arhythmia.

Several observations not included in this problem, but showing the effects of faradic and osmotic-chemical stimulation of various effective areas in the brain are listed on p. 349.

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BLOOD SUGAR, URINE SUGAR AND URINE PROTEIN IN EXERCISE

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Since the concentrations of sugar in blood and urine are modified by a large number of influences, it is not surprising that observations made by different investigators are not entirely harmonious. Thus Cannon (1) has reviewed the subject and has emphasized the importance of excitment in determining the blood level whether in rest or in exercise. He and Fiske found glycosuria in several Harvard football players, in non-playing substitutes and in an excited spectator after a game. After stair-climbing for 15 minutes, or until exhausted, Rakestraw (2) found increases in blood sugar in most of his 14 subjects, the average value rising from 95[1] to 131. On the other hand, when the exercise is moderate, Trimble and Maddock (3) have shown that blood sugar is unchanged. In runners finishing a marathon race Gordon and associates (4) and Best (5) found normal or low blood sugars. In 2 men completely exhausted, Best found values of 53 and 55. Depletion of carbohydrate reserves may account for such low values.

Our investigations[2] have been made along the following lines:

1. Concentration of blood sugar in exercise of varying intensity by subjects of different types.
2. Concentration of blood sugar in football men in relation to diet, length of play, environment and age.
3. Urine sugar and urine protein of football players.

Blood specimens were obtained from an ear-lobe with a capillary pipette and sugar was determined by the micro method of Folin and Malmros (6). Urine sugar was determined qualitatively with Benedict's solution or quantitatively by his method (7). Urine protein was estimated approximately by the degree of turbidity produced in the nitric acid test. Most of the

[1] Concentrations of sugar in blood and in urine are expressed in milligrams per 100 cc.

[2] The results reported here were obtained during the 1930 season. Records of similar observations obtained in former seasons by one of the authors (T. K. R.) and W. R. Ohler were largely destroyed in a fire.

blood samples after playing were obtained within 3 minutes of the time the player left the field. Urine samples were obtained in the locker room some time later.

Among subjects studied in the laboratory were 13 graduate students, selected because they had exhibited "emotional glycosuria" at the time of their examination by the medical adviser. One of these showed an increase in blood sugar from 96 to 172 during a run of 15 minutes at 5.8 miles per hour on a treadmill. None of the others showed more than a slight increase; the average for the other 12 was 103 before running and 95 after.

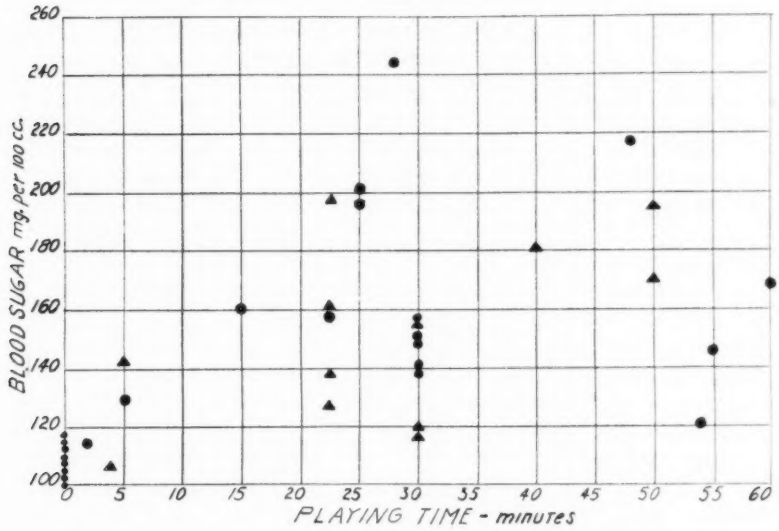

Fig. 1. Blood sugar in relation to length of play and presence of spectators. Circles represent varsity players in stadium games and triangles, second varsity players in games without spectators.

Two football players performed the same task. On the football field their blood sugar concentration had increased; in the laboratory it decreased. A great many observations on men accustomed to acting as experimental subjects indicate that, when excitement is absent, blood sugar concentration usually decreases after more than 15 minutes' exercise in the laboratory. The decrease tends to be greater, the more prolonged the activity, as found by Rakestraw.

Values for blood sugar in Harvard players are shown in relation to length of play in figure 1. Players before a game and even substitute players watching a game have normal blood sugars. The range before play began

was from 100 to 117 including 8 varsity players and 3 substitutes.[3] One player, withdrawn because of injury after only 3 plays, had a blood sugar of 115. Three men who played about 5 minutes had values of 106, 130 and 142. In the case of 16 men who played about half the game, values ranged from 117 to 244, with a mean value of 159 and a median value of 153. There is evidence of a slight decrease when men continue to the end of the game but the lowest value observed in such a man was 104. The absence of hypoglycemia is possibly related to the fact that most of the players are on a high carbohydrate diet and in addition often received 50 grams of glu-

TABLE 1

Urine sugar in relation to blood sugar

Concentrations in mgm. per 100 cc.

QUALITATIVE TEST FOR URINE SUGAR NEGATIVE OR DOUBTFUL		QUALITATIVE TEST FOR URINE SUGAR POSITIVE	
Blood sugar	Urine sugar	Blood sugar	Urine sugar
182	167	113	345
118	116	140	312
105	105	140	227
153	182	138	278
102	189	104	295
		155	357

TABLE 2

Urine protein in relation to length of play

NUMBER OF SUBJECTS	LENGTH OF PLAY	NUMBER OF SUBJECTS WITH POSITIVE PROTEIN TEST	PROTEIN IN URINE
	minutes		
9	5 to 15	4	0.8
11	15 to 20	10	1.7
9	30 to 45	8	2.1
13	45 to 60	13	2.8

cose as a candy or syrup shortly before a game. The occurrence of hyperglycemia is not dependent either on the special diet or on the extra sugar ingestion for the reason that some of the highest values have been found on second varsity men not on the special diet and not given sugar before the game and on certain varsity men with the special diet but not given extra sugar before the game.

[3] These observations on substitutes are surprising in view of the fact that glycosuria was found in substitute players by Cannon and Fiske. At the time their experiments were carried on it was the practice of the coaching staff to arouse the squad to a "fighting pitch" before the game. Possibly football is taken less seriously by players and spectators now.

In addition to observations on the Harvard teams, there has also been an opportunity at the Middlesex School and the Cambridge Latin School to study players 3 or 4 years younger. Results of a similar character were obtained. Their blood sugar concentration was, on the average, slightly less than that of Harvard players. The highest mid-game figure was 182 as contrasted with 244 in a varsity player. The highest value in a young player at the end of a game was 150, in a college player, 169.

Since men show little variation in blood sugar in exercise in the laboratory and an increase on the football field it is obvious that external conditions greatly influence this aspect of the internal environment. To the view of the spectator, the cheering crowd is a powerful stimulus to the player. The player affirms that he is dimly aware of this aspect of this environment; during play his attention is confined to the sphere of his activity. It is not surprising, therefore, that blood sugar concentration increases independently of the number of spectators. The sugar concentration in the blood of 13 varsity men after playing for more than 15 minutes had an average value of 168 (median, 151), while the average for 10 men playing on the second varsity before almost no spectators and with no cheering was 155 (median, 153).

A number of observations made on urine sugar are summarized in table 1. Since football playing may change the threshold value for sugar excretion it is difficult to estimate blood sugar from urine sugar. Thus one man had a blood sugar concentration of 182 with a normal urine sugar and on the other hand some of those with glycosuria did not have hyperglycemia. This latter anomaly occurred in men who played an entire game; probably their blood sugar concentration had been at a much higher level earlier in the game.

The concentration of protein in urine is definitely related to length of play. Thus, if one classifies the degree of turbidity in the nitric acid test as 0, 1, 2, 3, 4, and 5 the averages are as shown in table 2. The high incidence of proteinuria after play suggests that many cases of "orthostatic" proteinuria reported among college men may in fact be due to strenuous exercise preceding the medical examination.

SUMMARY

Hyperglycemia is uncommon in exercise with little or no emotional stress but common in exercise with emotional stress on the football field. In football players it is not much influenced by age within the range of 16 to 22 years, by diet nor by mass of spectators. Before the game begins blood sugar is normal and it appears to reach a peak when the game is half over. At the end of the game blood sugar may be normal while urine sugar is high, indicating that blood sugar has passed through a maximum. Inferentially if exercise were to continue (as in marathon races) hypo-

glycemia might result. Protein commonly appears in the urine of football players, increasing in concentration as play continues.

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THE TACHYCARDIA OF EXPERIMENTAL HYPERTHYROIDISM

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Several years ago, on the basis of his clinical observation, Plummer postulated that the tachycardia of hyperthyroidism was the result of direct action of the exciting agent on some part of the intrinsic cardiac mechanism, and that it was not dependent on the nervous system, particularly the sympathetic nervous system, a view current at this time For the last two years we have been attempting to devise methods of investigating the problem of the physiologic mechanism underlying the tachycardia of hyperthyroidism and to determine whether the clinical observations could be substantiated or refuted by direct experimentation.

The main difficulty encountered in a pharmacologic study of thyroxin on the heart is the slowness with which the drug acts. The tachycardia that follows the administration of thyroxin is not apparent until several hours after its administration. This delay in the demonstrable action of thyroxin makes it impossible to investigate by the usual method the action of a substance on the heart, such as direct application to the perfused heart. However, whether the increased heart rate of an animal that has received thyroxin persists after removal of the heart from the body can be investigated. It is also possible to study the effect of thyroxin on the transplanted heart. The results of such investigation, using these two general methods, form the basis of this report.

METHODS. *Perfusion of hearts.* In the first set of experiments heart-lung preparations were set up in the usual manner, with precautions to keep the procedure as close to the standard as possible. The preparations were made using normal dogs, and dogs that had been rendered hyperthyroid by the administration of thyroxin intravenously for several days prior to the experiment. The pulse rates of the preparations were counted during the perfusion period.

In another set of perfusion experiments, thyroxin was administered intravenously to normal rabbits. When the animals exhibited the usual symptoms of the effect of thyroxin, the blood-free hearts were excised and perfused after the manner of Locke and Rosenheim under carefully controlled and standard conditions. The hearts of normal rabbits were perfused under similar conditions. The pulse rates were counted during the perfusion period.

Transplantation of hearts. In order to approach the problem in another manner, we took advantage of a technic which we had recently developed. This consists of the transplantation of the heart from a small dog into the neck of a large dog. By means of the usual precautions, employed in the surgery of blood vessels, the heart was removed from a small dog after ligating all the main vessels except the aorta and the pulmonary artery. The aorta was united by means of blood vessel sutures to the carotid artery of the recipient and a branch of the pulmonary artery was similarly united to the external jugular vein of the recipient. This technic has been briefly described elsewhere (Mann, Markowitz and Priestley, 1931). It suffices to say at this point that hearts so transplanted contracted regularly long enough and at a reasonably constant rate for the purposes of the investigation. Electrocardiographic tracings were taken by direct leads from the base to the apex of the transplanted heart and its contractions were counted by palpation of the neck. Thyroxin was injected intravenously in several dogs that had been thus suitably prepared by transplantation of the heart into the neck, the pulse rates being counted before and after its administration.

RESULTS. *Perfused hearts from normal and thyroxinized dogs by means of heart-lung preparations.* The usual technic, with slight modification, was employed for heart-lung perfusions. Typical protocols of thyroxinized animals follow.

A male dog weighing 13.8 kgm. had received 10 mgm. of thyroxin intravenously each day for four days beginning January 31, 1929. The experiment was performed February 4. The pulse rate on the morning of the experiment, twenty-four hours after the last injection of thyroxin, was rather constantly 170 beats a minute in the standing position. A heart-lung perfusion was made. The circuit was completed at 11:10 a.m. The following observations were made:

TIME	TEMPERATURE OF BLOOD IN VEINS	HEART RATE EACH MINUTE
	°C.	
11:18	36.0	230
11:25	36.0	200
11:28	37.0	190
11:30	37.0	186
11:35	36.5	177
11:40	37.0	180
11:45	37.0	180
11:50	38.0	186
12:10	38.0	168
12:23	37.8	168
12:28	37.3	154
12:32	37.9	168
12:40	38.5	166
1:15	39.0	166
1:30	39.0	162
2:30	38.7	152
3:20	37.0	120
3:45	36.0	120

The experiment was discontinued.

A female dog weighing 11.8 kgm. was given 20 mgm. thyroxin intravenously at 2:30 p.m. April 16, 1929. The pulse rate (standing) was 100. April 17, the animal was very nervous; the pulse rate was 110 to 120 beats each minute. Fifteen milligrams of thyroxin were given intravenously. April 18, the animal showed extreme nervousness; the pulse rate was 150. A marked erythematous flush was evident on the ventral surface of the thorax and abdomen. Fifteen milligrams of thyroxin were administered. April 19, the animal was etherized, and a heart-lung preparation was made in the usual manner at 12:00 o'clock noon.

TIME	TEMPERATURE OF BLOOD IN VEINS	HEART RATE EACH MINUTE
	°C.	
12:05	36.0	182
12:10	36.0	160
12:15	36.0	167
12:20	36.0	174
12:30	36.0	166
12:40	35.5	157
12:50		156
1:15		160
1:35		164
1:45		166
2:00	36.5	171
2:20	35.0	157
2:35		160
3:05		157

The experiment was discontinued with the heart in good condition.

The rapid pulse rates were observed only in heart-lung preparations of dogs that had previously received thyroxin. They may be contrasted with the counts obtained in heart-lung perfusions prepared from normal dogs, of which typical protocols are given.

February 11, 1929, a heart-lung perfusion was made on a male dog weighing 5.0 kgm. The circuit was completed at 6:25 p.m.

TIME	TEMPERATURE OF BLOOD IN VEINS	HEART RATE EACH MINUTE
	°C.	
6:30	37	128
6:35	37	126
6:41	37	118
6:50	37	102
7:00	37	94

The experiment was terminated with the heart in good condition at 7:10 p.m.

February 22, 1929, a heart-lung perfusion was made on a male dog weighing 12.0 kgm. The circuit was completed at 1:15 p.m.

TIME	TEMPERATURE OF BLOOD IN VEINS	HEART RATE EACH MINUTE
	°C.	
1:40	36.5	140
2:00	37.0	140
2:20	36.0	136
2:50	36.0	132
3:35	36.0	108

The experiment was discontinued.

During several experiments we investigated the immediate action of thyroxin, and of blood from hyperthyroid animals on the heart rate of the heart-lung perfusion, with negative results. The observations were continued for four hours.

Occasionally a heart-lung perfusion made from a dog that had been given a large dose of thyroxin, gave pulse rates which were not higher than normal. Such exceptions always occurred in experiments in which cardiac failure occurred early. On the other hand, we did not observe rapid pulse rates in heart-lung perfusions made from normal dogs similar to those obtained after the administration of thyroxin.

Perfused hearts from normal and thyroxinized rabbits. A preliminary report of this phase of the investigation has been made (Priestley, Markowitz and Mann, 1931). Lewis and McEachern (1931) also recently published the results of their investigation on the rate of perfusion of the heart and auricles of thyroxinized rabbits. They found that the isolated heart and auricles of animals treated with thyroid continued to beat at a much faster rate (26 to 54 per cent) than similar preparations from normal animals. The results of their experiments and ours are in perfect agreement.

We employed the following method to study the effect of the administration of thyroxin on the rate of the subsequently perfused heart. Twenty rabbits were rendered hyperthyroid by the intravenous administration of thyroxin. Each animal was then etherized, and the heart was removed and perfused on the Locke and Rosenheim apparatus with Ringer-Locke's solution of constant hydrogen-ion concentration and temperature. The heart was observed until the rate and vigor of the contraction decreased and frequent determinations of the rate were made. Control observations were made of ten hearts obtained from ten normal rabbits. Two typical protocols follow.

An experiment was performed July 10, 1929, on a normal male rabbit weighing 1.5 kgm.

TIME	HEART RATE EACH MINUTE	TEMPERATURE OF PERFUSING FLUID
		°C.
9:50	150	36
9:57	132	36
10:18	100	36
10:30	100	36
11:12	95	36
11:58	96	36
1:40	90	36
4:00	90	36

The experiment was discontinued with the heart in excellent condition.

An experiment was performed July 17, 1930, on a male rabbit weighing 2.4 kgm. Two injections each of 2 mgm. thyroxin had been administered July 10 and July 12, respectively.

TIME	HEART RATE EACH MINUTE	TEMPERATURE OF PERFUSING FLUID
		°C.
1:45	186	36
2:05	188	36
2:30	207	36
3:06	186	36
3:37	179	36
3:52	180	36
4:22	163	36
4:37	163	36

The experiment was discontinued with the heart beating with fair vigor.

Occasionally a heart from a thyroxinized rabbit pulsated at a rate within normal limits. The contraction of such hearts decreased soon after the perfusion was begun. It was found that the addition of thyroxin, dissolved in alkalinized solution of sodium chloride, to Locke's solution that is being perfused through a normal rabbit's heart has no appreciable influence on rate over a period of nine hours.

Observations on the transplanted heart. Careful observations were made of the rate of beat of the transplanted heart for two or three days before administering the thyroxin to the recipient. This was necessary to determine the basic rate of the transplanted heart. Thyroxin was then injected intravenously. Usually no effect was noted until about twenty-four hours had elapsed. Then the rate of the transplanted heart increased. Sometimes the increase of the heart rate of the recipient was slight with the amount of thyroxin used. A typical protocol follows.

The heart of a male puppy weighing 20.8 kgm. was transplanted June 22, 1930. Coronary circulation was established at 10:00 a.m. The heart started to beat immediately and continued beating regularly until it stopped on the morning of June 30. The preparation was satisfactory throughout. At 2:45 p.m., June 25, 20 mgm. of thyroxin were injected intravenously. The following rates of heart beat were taken from electrocardiograms until June 29, when the rate was estimated by palpation only.

DATE	TIME	RECIPIENT	TRANSPLANT
6–23–30	p.m.	108	132
6–24–30	a.m.	126	188
	p.m.	124	162
6–25–30	a.m.	114	156
	p.m.	126	180*
6–26–30	p.m.	132	222
6–27–30	a.m.	114	222
6–28–30	p.m.	120	220
6–29–30			Approximately 180

* 20 mgm. thyroxin.

COMMENT. It would appear from these experiments that it is not necessary to involve the central nervous system and the sympathetic nervous system in explaining the tachycardia which follows the administration of thyroxin. The result of this investigation indicates that the cause for the increased heart rate after thyroxin is peripheral. Not only does the tachycardia following the administration of thyroxin appear to be independent of the central nervous system, but it actually seems that the central nervous system exerts a restraining influence on such tachycardia because the denervated transplanted heart responded with an increase in rate after thyroxin whereas the innervated heart of the recipient only increased slightly or not at all.

SUMMARY

A series of experiments was performed in which it was shown that the heart rate of perfused hearts of thyroxinized rabbits and the heart-lung preparation of thyroxinized dogs is considerably greater than normal. Similarly, when the heart of a small dog is transplanted into the neck of a large dog by means of anastomosis of blood vessels the administration of thyroxin evokes definite tachycardia of the transplanted heart. It appears that the tachycardia of experimental hyperthyroidism is not dependent on the central nervous system, but on a peripheral mechanism.

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